Vol. 25 No. 2

April - June, 1959

THE BOTANICAL REVIEW

Interpreting Botanical Progress

AUXINS
J. van Overbeek

MITOSIS AND METABOLIC ORGANIZATION
Herbert Stern

CYTOTAXONOMY OF NICOTIANA. II
T. H. Goodspeed and Mildred C. Thompson

FUTURE CONTENTS
ON INSIDE BACK COVER

Published Quarterly

Second class postage paid at Plainfield, New Jersey

Printed in U.S.A.

THE BOTANICAL REVIEW

Founded in 1935 by

HENRY ALLAN GLEASON and EDMUND H. FULLING

Managed, edited and published at

The New York Botanical Garden

by

EDMUND H. FULLING

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Articles are obtained primarily by invitation. Unsolicited manuscripts are also welcome. In all cases, however, the editor reserves the right to accept, reject or suggest revisions in submitted material. All manuscripts should be on double-space typewritten pages, with references and citations in accordance with the customary style of THE BOTANICAL REVIEW.

Missing numbers can not be supplied without charge unless notice is received within two months after appearance of issues concerned.

All correspondence should be addressed to THE BOTANICAL REVIEW, NEW YORK BOTANICAL GARDEN, NEW YORK 58, N. Y.; not to Interstate Printing Corporation, Plainfield, N. J.

*Price of this issue (Vol. 25, No. 2): $2

THE BOTANICAL REVIEW

VOL. 25 APRIL-JUNE, 1959 NO. 2

AUXINS[1]

J. VAN OVERBEEK
Shell Development Company
Agricultural Research Division
Modesto, California

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[1] Material in this article was delivered as a series of lectures for the University of Nebraska during April, 1957.

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INTRODUCTION

The first discussion of auxins in The Botanical Review was written by Went in 1935. The field was very new at that time. Only seven years had elapsed since Went (1928)[1a] had isolated a growth-regulating principle, auxin, from oat coleoptile tips, and its chemical identification had barely been achieved. It was already correctly understood during this early period that auxin-induced growth is a consequence of auxin-induced softening of the cell wall (Heyn, 1931).

In a supplement Went (1945) reported on the developments during the decade since 1935. At that time it was recognized that a multitude of chemicals possess auxin activity, and that these have minimum structural features in common. Further, it had become known that auxins affect a great many physiological processes.

In the period between the 1945 review and 1957 so much has been added to our knowledge that it has become necessary to write an entirely new review.[2] As I see it, three outstanding events characterize these 12 years. Not of least importance is the wide commercial use of synthetic auxins as herbicides and as growth regulators. Auxinology has made a significant contribution to modern agriculture, a contribution equal in significance to that of the introduction of fungicides and insecticides. Secondly, fundamental knowledge has increased because of the adaptation of paper chromatography to auxin research. As a result of this, we now clearly recognize indoleacetic acid and its closely related derivatives as the principal naturally occurring auxins in higher plants. Finally, during very recent years gibberellins and kinins have been added to the list of chemically well defined growth regulators. There is little doubt that gibberellins are naturally occurring in higher plants and may be considered hormones. Gibberellins are different from auxins, and the work of Phinney (200) on dwarf corn, and of Whaley (292) on roots indicates that the responses of auxins and gibberellins are conditioned by different genes. Gibberel-

[1a] In deference to the author's manner of citation, the style customarily observed in the REVIEW is not followed in this article, in that numbers within parentheses refer, sometimes, to years of publication, at other times to citations in the bibliography. In the great majority of cases, if not in every instance, references cited by authors' names and years of publication are to be found in the fourth group of citations [Ed.].

[2] This does not mean that reviews have not been published elsewhere. Indeed, 26 reviews published since 1950 have been listed in the front part of the literature list of this article.

lins strongly promote the growth of tissues during growth phases that auxins do not. On the other hand, auxin inhibits such gibberellin-controlled growth (267). It appears, therefore, that in the future auxins must be studied in conjunction with gibberellins. Auxin is therefore no longer "the" plant growth-hormone as the title of the earlier reviews indicated. Even though kinins have not yet been shown to occur in higher plants, this would seem most reasonable. Skoog et al, the discoverers of these growth factors, have shown that kinetin causes nuclear and cell division, but only in the presence of auxin (227). The picture that develops is one of an interplay of hormones which determines the physiological response of an organ or a tissue.

In this article I have attempted to review the present status of auxin research. I have tried to do this in such a fashion that the student unfamiliar with this field can use the article as a basis for further studies. Old facts have been combined with new knowledge and as a result a number of new ideas have arisen. I hope these will serve as working hypotheses for further experimentation and aid in the understanding of the growth process in plants.

DISCOVERY

Pin-pointing the origin of an idea back in history is difficult, if not impossible. It is more a question of taste than anything else. As far as I am concerned, I like to consider the publication of Darwin's "The Power of Movement in Plants" (1880) as the starting point of modern plant hormone research. In that publication, Charles and his son, Francis, described the important discovery that inside plants a stimulus is transmitted from one part to an adjoining part. The tips of the cotyledons of *Phalaris* and *Avena,* and the upper part of the hypocotyls of *Brassica* and *Beta* are highly sensitive to light. Some influence is transmitted from these upper to the lower parts, causing the latter to bend towards light. "This influence is also transmitted beneath the soil to a depth where no light enters. In the case of the radicles of *Sinapis alba,* sensitiveness to light also resides in the tip, which, when laterally illuminated, causes the adjoining part of the root to bend apheliotropically" (p 566-567). Being a naturalist with wide zoological experience, Darwin observed that there is a most striking resemblance between plants and animals in the "localisation of their sensitiveness, and the transmission of an influence from one

excited part to another which consequently moves. Yet plants do not of course possess nerves . . .".

Darwin's contemporary, Julius Sachs, in his "Vorlesungen über Pflanzen-Physiologie" (1882), objected to Darwin's views rather crudely: "Es ist bei derartigen Untersuchungen an Wurzeln nicht nur grosse Vorsicht, sondern auch langjährige Übung und allseitige Bekanntschaft mit pflanzenphysiologische Dingen nöthig, um nicht in die gröbsten Irrthümer zu verfallen, wie es Charles Darwin und seinem Sohne Francis ergangen ist, die auf Grund ungeschickt angestellter und zudem falsch gedeuteter Versuche zu dem ebenso wunderlichen wie sensationellen Ergebniss gelangten, dass der Vegetationspunkt der Wurzel ähnlich wie das Gehirn einer Thieres die verschiedenen Bewegungen an der Wurzel beherrsche" (p. 843).[3]

Such unjustified criticism by this continental authority on plant physiology may have contributed in delaying the search for the "matter in the upper part which is acted on by light and which transmits its effects to the lower parts" (p. 486, Darwin). It was not until 50 years later (Went, 1928) that the "matter" of which Darwin spoke was identified as the plant hormone which we now call "auxin," the main subject of our discussion.

Although the last word has not been spoken on the mode of action of phototropic bending, a simplified interpretation may be as follows: In the tip of the coleoptile tryptophan (I)[4] is converted into indoleacetic acid (II); this indoleacetic acid (IA) is a plant hormone which flows down the coleoptile. While it does so, it stimulates the growth of the young cells it meets on its path. When the coleoptile tip is unilaterally illuminated, the hormone, somehow, becomes unilaterally distributed (Went, 1928; Briggs et al, 1957; Gordon, 1957). One finds less hormone on the illuminated than on the shaded side, with the result that a wave of hormone moves down the plant which

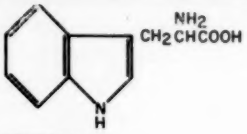
[3] Sachs' own views on phototropism reflect a surprising lack of clear thinking, as the following quotation (p. 851) shows: "meine soeben angedeuteten Uberlegungen führen mich nun zu dem Schluss, dass auch bei den heliotropischen Krümmungen es nicht auf eine *Differenz der Intensität* der enwirkenden Kraft auf entgegengesetzten Seiten des Organs ankommen möchte, dass vielmehr die heliotropische Wirkung dadurch hervorgerufen wird, dass die Lichtstrahlen das Pflanzengewebe oder auch nur einzelne Zellen in einer *bestimmten Richtung* durchsetzen".

[4] Structural formulae, names and abbreviations of auxins and related materials are given in Table I.

TABLE I

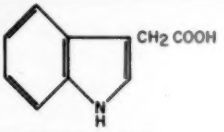
STRUCTURAL FORMULAE, NAMES AND ABBREVIATIONS OF AUXINS AND RELATED MATERIALS*

I



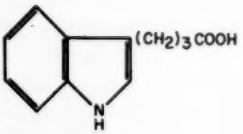
TRYPTOPHAN

II
IA



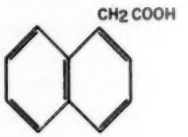
3-INDOLEACETIC ACID

III



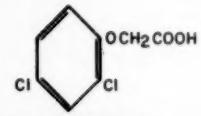
3-INDOLEBUTYRIC ACID

IV
NA



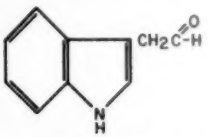
1-NAPHTHALENEACETIC ACID

V
2,4-D



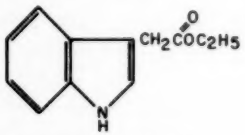
2,4-DICHLOROPHENOXY ACETIC ACID

VI



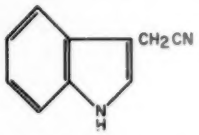
3-INDOLEACETALDEHYDE

VII



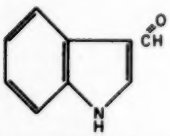
ETHYL-3 INDOLEACETATE

VIII
IAN



3-INDOLE ACETONITRILE

IX



3-INDOLECARBOXALDEHYDE

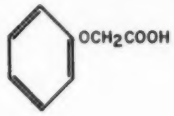
X
PA

PHENYLACETIC ACID

* Names are those recommended by the American Chemical Society, as used in Chemical Abstracts. Straight-line formulae of numbers XXI to XXV are given in Table II on page 307.

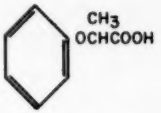
TABLE I (Cont.)

XI
POA



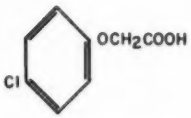
PHENOXYACETIC ACID

XII



2-PHENOXYPROPIONIC ACID

XIII

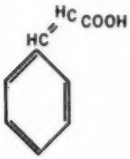


4-CHLOROPHENOXYACETIC ACID

XIV

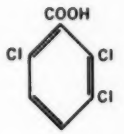
TRANS-CINNAMIC ACID

XV



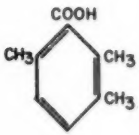
CIS-CINNAMIC ACID

XVI



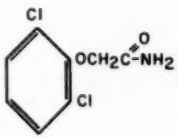
2,3,6-TRICHLOROBENZOIC ACID

XVII



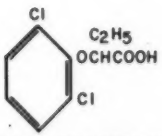
2,3,6-TRIMETHYLBENZOIC ACID

XVIII



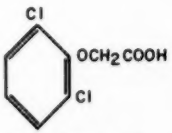
2,6-DICHLOROPHENOXYACETAMIDE

XIX



2-(2,6-DICHLOROPHENOXY)
BUTYRIC ACID

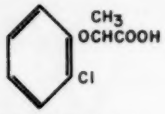
XX
2,6-D



2,6-DICHLOROPHENOXY-
ACETIC ACID

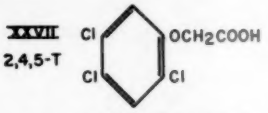
TABLE I (Cont.)

XXVI



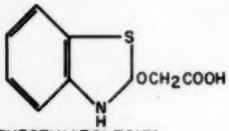
2-(2-CHLOROPHENOXY) PROPIONIC ACID

XXVII 2,4,5-T



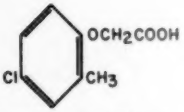
2,4,5-TRICHLOROPHENOXY-ACETIC ACID

XXVIII



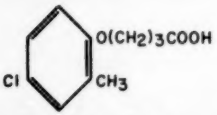
2-BENZOTHIAZOLEOXY-ACETIC ACID

XXIX MCPA



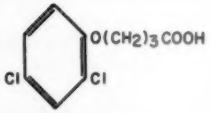
2-METHYL-4-CHLOROPHENOXY-ACETIC ACID

XXX MCPB



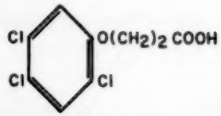
2-METHYL-4-CHLOROPHENOXY-BUTYRIC ACID

XXXI 2,4-DB



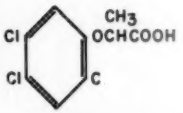
2,4-DICHLOROPHENOXY-BUTYRIC ACID

XXXII TP



2,4,5-TRICHLOROPHENOXY-PROPIONIC ACID

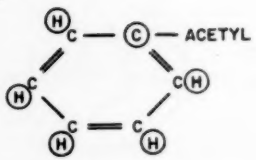
XXXIII SILVEX



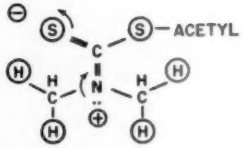
2-(2,4,5-TRICHLOROPHENOXY) PROPIONIC ACID

TABLE IB

X



XXI



has a higher concentration in the back than on the front. Consequently, as the hormone promotes growth, it promotes growth more on the rear than on the front, which in turn causes the seedling to bend toward the light. This hormone wave continues to pass downward toward the base, even though the base might never have been exposed to light.

The pigment in the tip which absorbs the light is not known with certainty. It may involve carotene, it may involve a pigment system with more than one pigment. It is also by no means certain that indoleacetic acid is the only hormone involved. The study of phototropism with modern techniques is still a fruitful subject for research.

DEFINITIONS

Indoleacetic acid is a plant hormone, that is, a chemical made by the plant which controls growth and other processes. A number of terms must be defined at this time. After many years of study a committee of the American Society of Plant Physiologists has made certain recommendations regarding nomenclature of plant hormones, auxins and growth regulators. These recommendations were accepted by the Society on April 6, 1953. They are published in Plant Physiology, the official journal of the Society, in Vol. 29:307, 1954. The recommended definitions follow:

(Plant) Regulators: organic compounds, other than nutrients,[5] which in small amounts promote, inhibit or otherwise modify any physiological process in plants.

(Plant) Hormones (Synonym: Phytohormones): regulators produced by plants, which in low concentrations regulate plant physiological processes. Hormones usually move within the plant from a site of production to a site of action.

Growth Regulators (Synonym: Growth substances): regulators which affect growth.

Growth Hormones are hormones which regulate growth.

Flowering Regulators are regulators which affect flowering.

Flowering Hormones are hormones which initiate the formation of floral primordia, or promote their development.

Auxin is a generic term for compounds characterized by their

[5] Nutrients are here defined as materials which supply either energy or essential mineral elements.

capacity to induce elongation in shoot cells. They resemble indole-3-acetic acid in physiological action.

Auxins may, and generally do, affect other processes besides elongation, but elongation is considered critical. Auxins are generally acids with an unsaturated cyclic nucleus or their derivatives.

Auxin Precursors are compounds which in the plant can be converted into auxins.

Anti-Auxins are compounds which inhibit competitively the action of auxins.

The definition of auxin is based on the physiological reaction pattern of indoleacetic acid. This is realistic, and for this reason has proven itself useful in practice. For instance, it has allowed a clear distinction between auxins and gibberellins, and has thus prevented much confusion. Although gibberellins do cause cell elongation, they promote cell elongation under conditions where IA usually does not. In addition, IA prevents development of axillary buds, while gibberellic acid (GA) promotes it. IA generally inhibits flowering, while GA promotes it. GA differs quite markedly from the auxins in the effects it produces (Kato, 1955, 1958; Brian, 1957).

Although a gibberellin is not an auxin, it is a growth regulator; and if it functions in the plant's normal physiology, it can also qualify as a hormone.

The term "growth regulator" has been used since the early days of auxin research. In the preliminary publication of 1926, announcing the discovery of the isolation of the substance which we now call "auxin," Went speaks of "groeiregelaars" (growth regulators).

DEVELOPMENT

Isolation of the first plant hormone was achieved on the European Continent some 50 years after Darwin had anticipated it. In 1926, F. W. Went, then at Utrecht, succeeded for the first time in separating the hormone from the tip of oat seedlings. It was not chemically identified at that time. However, Went had also invented a simple bioassay, known as the Avena test, which measures the plant hormone accurately. Chemical isolation could now proceed, as the Avena test could tell the chemists which fractions were enriched and which were devoid of hormone. In 1934, Kögl and Haagen-Smit, also at Utrecht, completed the chemical isolation and identified indoleacetic

acid as an auxin, but it was by no means clear at that time that IA is the major auxin in higher plants. This question will be further discussed in the third chapter.

Synthesis of indoleacetic acid was a relatively easy process, and soon plant physiologists, the world over, could buy the crystals from chemical supply houses. Easy availability of pure auxin has helped much in the rapid development of the auxin field.

It may be well to review briefly the developments in this country. Auxin research started at Caltech, which in 1920 had been transformed from a polytechnic school into a research institute under the leadership of the Nobel Prize Winner Robert Andrew Millikan. In 1928 Thomas Hunt Morgan, later also to become a Nobel Prize Winner, started the biology department. Morgan added a plant hormone physiologist from the Dutch research team to his staff to start a nucleus of auxin work in California. This man was Dr. H. Dolk. However, soon after his arrival he met an untimely death in an automobile accident in the Southern California Desert. Dolk had two collaborators, a young biochemist, Dr. K. V. Thimann, and a graduate student, James Bonner. Thimann worked on the isolation of the growth hormone from fungus culture. He identified it as indoleacetic acid in 1935. In the meantime, F. W. Went, Folke Skoog and myself had joined the Caltech group, and from that nucleus hormone research branched all over the country. Avery (Brooklyn Botanic Garden), Mitchell (USDA), Gustafson (Michigan), Riker (Wisconsin), to name but a few of the earlier workers, all spent time at Caltech to acquaint themselves with the new techniques.

Zimmerman and his associates at the Boyce Thompson Institute soon introduced and developed chemicals different from indoleacetic acid which, however, caused physiological reactions in plants similar to IA. These materials could be used in agriculture and horticulture because they were more stable in the plant than IA. First indolebutyric acid (III) was introduced in 1935. This is a simple higher homolog of IA. Soon, however, naphthaleneacetic acid (IV) was found to have a strong auxin action. And, in 1942, they introduced 2,4-dichlorophenoxyacetic acid (V), widely known as 2,4-D. This auxin is chemically very different from the original IA, and it has a physiological action 100 times as potent. All these synthetic materials may be classified as auxins. They also are growth regulators, but "auxins" is a

more specific term. They are not hormones because they do not function in the normal physiology of the plant.

AUXIN-REGULATED CELL GROWTH

CELL ELONGATION OF COLEOPTILES

As soon as auxin was isolated from coleoptile tips and then applied to young coleoptile stumps, it was realized that this homone controls cell elongation (Went, 1928). Even before auxin was chemically identified, the mechanism by which auxin induces cell elongation was already worked out. It was shown that under the influence of auxin the cell walls soften, thereby allowing osmotic pressure inside the cell to stretch them (Heyn, 1931; Heyn and van Overbeek, 1931). This older work has been entirely substantiated in recent years (48).

Growth of cells by elongation is essentially an inflation of the cells with water. Therefore, one of the first effects of auxin is an increased intake of water by the tissue. How this water is taken up has been the subject of much discussion during the past quarter-century. It has been suggested from time to time that auxin might increase the permeability of the cell to water. That this is not so, at physiological concentrations, has been shown in an elegant fashion by Ordin and Bonner (1956) with deuterium hydroxide. Indoleacetic acid had no effect on the diffusion of DHO into or out of *Avena* coleoptile sections. It has also been suggested from time to time that, in addition to water taken up by osmosis, there is a non-osmotic intake of water by cells. The work of Ordin et al. (190) has made this unlikely for *Avena* coleoptile tissue. We must conclude, therefore, that auxin-induced water uptake by *Avena* coleoptiles follows osmotic principles (48).

In osmotic equilibrium, the suction pressure (SP) of a cell equals the difference between the osmotic pressure of the cell contents (OP) and the wall pressure (WP): $SP = OP - WP$

In order for a cell to take in water, its SP must increase. This can be done either by increasing OP or decreasing WP. In recent years it was shown again that in elongating cells there is no increase in the concentration of osmotically active materials inside the cell (147, 150). Consequently, there is no other alternative than that the wall pressure is decreased as a result of auxin treatment. This reduced tensile strength of the wall has been demonstrated directly by mechanical

stretching tests (Heyn and van Overbeek, 1931; Tagawa and Bonner, 1957). Auxin, therefore, causes elongation by reducing the cell wall pressure.

The next question is: How can auxin bring about the softening of the primary cell wall? In order to answer this, one must consider the fine structure of the primary wall. This structure could be compared best to a wall of reinforced concrete. The mass of the wall consists of pectins and hemicellulose, while there is a reinforcing network of cellulose fibrils. The cellulose in the wall amounts to only about 5% of the fresh weight (91, 92). The cellulose fibers are deposited by the cytoplasm transversally to the direction of growth. If the cells elongate rapidly, the fibers become reoriented axially. They are pulled this way passively like a bunch of filamentous algae on a glass plate (133). Cellulose deposition takes place during elongation, and apparently over the entire cell surface (218).

Knowing the predominance of pectins in the wall and recognizing the relatively non-crystalline nature of these hydrated materials, it occurred to the writer in 1939 (256, p. 660) that the increase of cell wall plasticity by auxin could be caused by a weakening of these pectic substances. The role of the cellulose micelles would be that of strengthening the cells tangentially and preventing growth laterally. This view has gained favor in recent years and appears now well supported by experimental evidence. Cleland (1957) gave labelled glucose and methionin to auxin-treated coleoptile sections, and found incorporation of both into the methyl ester groups of pectin. He concludes that the increase in rate of pectin methyl ester incorporation is an integral part of the cell-wall loosening process. Tagawa and Bonner (1957) found a striking decrease in plasticity and elasticity in coleoptiles that were pre-treated with calcium or magnesium solutions. Potassium, in addition to auxin, softened the cell wall. These facts suggest the coleoptile cell wall as made up of a network of pectic chains crosslinked at intervals by ionic bonds in which calcium ions bind carboxylate ions of adjacent chains. Replacement of calcium ions by potassium ions would weaken such ionic interaction between pectic chains and produce a more plastic cell wall. Ca^{45} was readily exchanged for K in a pectate gel in vitro as well as in the coleoptile. Under the influence of auxin, calcium bonds are broken by methyl esterification (192.)

CELL ENLARGEMENT

Isodiametric growth of cells, as in potato tubers, fruit flesh and pith cells, is also promoted by auxin. As in cell elongation, water uptake is involved. It has been known for 20 years that auxin-treated potato tissue takes up a considerable amount of water. Because this process is so much slower than the response to auxin in coleoptiles, the auxin-induced water uptake by potato tissue was for many years viewed apart from growth. The excellent work of Carlier and Buffel (1955) has clarified this problem. It demonstrated that auxin-induced water uptake of the potato is the result of growth by cell enlargement. During this process cell wall materal is constantly laid down, but under the influence of auxin there occurs a shift in cell wall composition: relatively less cellulose, but relatively more pectin is laid down. The extensibility of the cell wall to mechanical stretching was higher in the auxin-treated tuber tissue than in the untreated control.

Neither an increased concentration of osmotically active material in the cells (258), nor an increased permeability of the cells to water (251) can explain the auxin-induced water uptake in potato tissue. The relative increase in wall extensibility by auxin can, therefore, be ascribed to the fact that softer cell wall materials are synthesized during auxin treatment. Wells (284) has recently shown that in pith tissue also there is a large increase in pectin precursor under the influence of auxin. Bryan and Newcomb (1954) were among the first to measure a change in pectic substances in tobacco pith cultures as a result of auxin treatment.

Newcomb and Siegesmund (1957) have attempted to observe auxin-induced changes in the pith cell wall with electronmicrography. It was found that the cell walls are traversed by dense bands which are much wider in the auxin treated cells. The bands suggest plasmodesmata, but their electron-dense contents have not yet been identified. The possibility that calcium salts of callose are involved is being considered. Callose has been studied in recent years by Currier (1957), and it appears to be more abundant in cells and tissues than formerly thought. It was also noted on the electronmicrogram that the auxin-treated cells possess a larger number of granules. These might be associated with the increased enzyme activity in such cells. Newcomb was among the first to point out that ascorbic acid oxidase activity is increased in auxin-treated tissues. Ascorbic acid oxidase is a "sur-

face enzyme" as it is found in association with cell wall fragments (49). Whatever the function of ascorbic acid oxidase in the plant's metabolism, it is definitely not a terminal oxidase (49).

However, it may well be that the high ascorbic oxidate activity of auxin-rich cells is geared to cell enlargement, with the specific function of regulating the oxidation-reduction potential. It is recognized (Stern, 1955) that nuclear division proceeds under anaerobic conditions. Ascorbic acid maintains the reducing conditions required for such early phases of cell growth. It induces chromosome division (219). Soluble sulfhydryl groups are present in relatively high concentrations in cells which are undergoing division. And, flavin-catalyzed reduction of disulfide groups in the wall protein of dividing yeast cells maintains the cell wall plasticity necessary for the completion of cytoplasmic division (Stern, 1956). Cell elongation by contrast is a highly aerobic process. For this reason the oxidation potential of the environment must change during the growth of a cell. The highly reducing medium must give way to an aerobic one by the time the cell is ready to enlarge. It may be the function of ascorbic acid oxidase to bring about this change by oxidation of ascorbic acid.

At this time the energy requirements for growth might be mentioned. Growth is an endergonic process; it requires energy. An endergonic reaction occurs only if it is coupled to an exergonic reaction. Respiration, and in a sense photosynthesis, are the exergonic processes in plants. In plants as well as in animals, the common reactant that couples exergonic and endergonic reactions is adenosine triphosphate (ATP). ATP is generated by respiration and photosynthesis and is utilized in growth. As a consequence the rate of respiration is governed by the need for ATP in the growth reaction (35, 154). The increased rate of respiration found after auxin treatment is thus a consequence of the growth reaction and not due to the presence of auxin itself (33, 45, 90).

CELL ELONGATION IN ROOTS

Much of our knowledge of the action of auxins has been obtained from the study of coleoptiles. The growth physiology of coleoptiles has become a sort of standard by which the growth of other organs is often compared. Roots have a pattern of growth which seems diametrically opposed to that of the coleoptile. For instance, the root is positively geotropic, while the coleoptile is negatively geotropic. Roots

generally respond to auxins with growth inhibition, while coleoptiles are generally promoted by them. Root elongation even has a negative temperature coefficient. Roots elongate more at lower temperatures. Careful analysis of root growth, however, reveals that the differences between root and shoot growth are not as large as they seem at first sight. Thus Burström (1957) has shown that this negative temperature coefficient of root elongation is not a matter of the growth rate being lower at a higher temperature, but simply a result of a shorter period of elongation at the higher temperatures. At the higher temperatures, cell wall material is formed faster, the wall loses its extensibility faster, and therefore must stop elongating earlier. These two processes, wall elongation and deposition of rigid wall materials, occur in the coleoptile as well as in the root. However, in the root the latter process is relatively more emphasized. It seems to the reviewer that in this emphasis on formation of rigid cell wall materials in the root we have the clue to the understanding of root elongation.

The fine structure of root cells does not seem to differ much from that of other cells. There is a pattern of transversally oriented cellulose strands in the primary root wall with non-cellulose areas between the cellulose (173, 215). The non-cellulose material is probably pectin (70). Plasmodesmata cross the cell walls, and mitochrondria, microsomes and an endosplasmic reticulum are present in root cells (132).

Burström recognizes two phases in root cell elongation. The first one is a plastic stretching of the cell wall, the second phase involves wall formation. He assumes that the first phase is promoted by auxin, and discovered the interesting fact that auxins will promote root cell elongation consistently provided the cells are deficient in calcium. The second phase of elongation, active wall formation, is promoted by calcium and "is probably inhibited by IA".

The observation that in the relative absence of calcium, root elongation is promoted by auxin, is in complete agreement with the observations on coleoptiles (68, 243). In shoots as well as in roots (70), calcium hardens the walls by the formation of ionic bonds between adjacent pectic chains. However, even though it has been shown by Burström that under certain conditions auxin is definitely capable of promoting elongation of root cells, it still does not necessarily follow that in nature the relaxation of the root wall is brought about by auxin (253). Whaley and Kephart have shown that roots can elongate

under the influence of gibberellin. Since gibberellins do not inhibit root growth (55) and seem especially capable of bringing about cell elongation at low temperatures (297), one wonders whether a material such as gibberellin rather than auxin would not be nature's choice for bringing about the first phase of root elongation. This possibility has further intriguing consequences which will be discussed below under GA/IA interactions.

Recently, Perlis and Nance (1956) have demonstrated that auxin enhances the synthesis of cellulose in wheat roots. This indicates that the second phase of root elongation, formation of wall material, is not inhibited but is promoted by auxin. If one now remembers that in root elongation the process of wall deposition is much more prominent than in coleoptiles, the physiology of root growth becomes at once much clearer. It is the result of two virtually opposing processes—the first phase under normal conditions may not be controlled by auxin at all, the second phase is promoted by auxin.

As has already been suggested by Burström, it would seem that the notion that the physiology of the root is controlled by supraoptimal auxin concentrations should be abandoned; and it now appears, in favor of the idea that auxin promotes cell wall deposition, thereby hardening the root wall and virtually "putting the brakes" on elongation.

CELL DIVISION AND DIFFERENTIATION

Although initially auxin was considered a hormone for cell elongation, it is now recognized that auxin also can induce cell multiplication. The use of auxins had a decisive role in tissue culture technique, since the tissues of most dicots proliferate only in the presence of these growth substances (Gautheret, 1955). Tumor tissues, by contrast, can generally be grown in vitro without auxin, as these produce considerable quantities of auxin (148). Some dicot tissues require additional growth factors. Pith tissue of tobacco is such a tissue. Skoog et al. (81, 100) have shown that some cell division can be induced with IA. However, if IA was given in the presence of kinetin, mitosis and cell division resulted. Kinetin itself, without IA, did not promote mitosis or cell division, although it caused diphosphopyridine nucleotide (DNA) synthesis. For this tissue the full cycle of cell multiplication—DNA synthesis, mitosis, cell division—requires both auxin and kinetin. Root formation on these undifferentiated tissues can

be induced by a low kinetin/IA ratio; bud development by a high ratio.

Auxin also appears to have a role in xylem formation. Jacobs has presented evidence showing that in normal development as well as in regeneration auxin controls xylem differentiation (136-139). Wetmore agrees with these findings (291).

An interesting link between auxin and xylem resides in the biochemistry of lignin formation. Lignification of the walls of xylem elements is a well known fact. Eugenol is a lignin procursor. According to Siegel (221, 222), eugenol is oxidized by peroxidase, using H_2O_2 as a substrate. This same peroxidase may be activated in the tissues under influence of indoleacetic acid. As will be discussed below, Galston contends (93, 95, 96) that one of the enzymes which is involved in the destruction of IA is peroxidase, and that this peroxidase is an "induced" enzyme. In other words, peroxidase is formed as a consequence of the presence of indoleacetic acid in the tissues. Since lignin formation is one of the functions of peroxidase, it would seem logical that IA causes lignin formation.

BUD GROWTH AND AUXIN-GIBBERELLIN INTERACTION

Auxin controls the growth of lateral buds only in a negative sense. It inhibits development of the lateral buds, a fact discovered by Thimann and Skoog in 1933 (250). The inhibitory action of the terminal bud in keeping the lower buds on the stem in dormant condition is undoubtedly due to its production of auxin. The phenomenon is known as "apical dominance" and was very recently reviewed by Gregory and Veale (114).

During the past year it has become evident that gibberellic acid can break apical dominance, that is, it can override the action of auxin and make all lateral buds develop. Brian et al. (54) showed that GA stimulated the growth of lateral shoots from the lower leaf axils of the main stem of peas. Marth et al. (169) showed that GA treatment induced early development of vegetative buds. Cooper (69) sprayed GA on dormant grapefruit trees and observed that two weeks later all buds started to develop, while the untreated controls did not start until a month later. Lippert et al. (158) found that GA increased sprouting of potato tubers; even the lateral buds on the branches sprouted. All this points clearly to the conclusion that gibberellins are powerful growth promoters for buds.

We are therefore, at present, in the favorable position of possessing regulators by which one can at will either suppress or promote the growth of a bud. That auxin antagonizes gibberellin-induced growth became very clear recently from experiments in our laboratory (267). The basal embryonic portion of the shoot which is enclosed in the *Avena* coleoptile does not grow at all under the influence of auxin; in fact, auxin inhibits its growth. This embryonic shoot does, however, respond strikingly to gibberellic acid in concentrations as low as 0.005 ppm. The increase in growth is linearly proportional to the log of the GA concentration. However, if as little as 0.05 ppm of IA is present, the entire GA effect (even by GA concentrations as high as 0.5 ppm) is strongly inhibited. The base of the embryonic *Avena* shoot enclosed in the coleoptile can be considered a bud. It grows under influence of gibberellic acid, but is kept from developing by indoleacetic acid. It would seem that from this simple test we have learned to understand the mechanism of bud growth and apical dominance: the bud grows under influence of gibberellin but auxin suppresses this gibberellin activity. (See ADDENDA for proof of this view).

The action of this inhibitory effect of auxin on gibberellin may also explain several other hitherto puzzling phenomena. For instance, Kuraishi and Hashimoto (152) found that a pea leaf attached to the plant could not be stimulated by GA. When the same leaf was detached it responded well to GA. The isolated leaf was removed from the flow of auxin of the intact plant, thereby allowing expression of the gibberellin effect. Another possible aspect of the antagonistic effect of IA on GA is in flowering. It is not impossible that gibberellin functions in the natural process of flower formation of many plants. The successful flower induction by gibberellin in a large number of long-day plants suggests this. It would be expected on this basis that auxins would inhibit flowering. This indeed is generally the case. Another reason for the inhibition of root growth by auxin, in addition to those mentioned in an earlier section, is that it represents another aspect of a gibberellin effect inhibited by IA. It will be recalled that we considered the possibility that the first phase of root growth was GA-, not IA-, controlled. In a section below dealing with auxin-controlled fruit drop, the thesis will be presented that at certain stages in the life of a young fruit its growth depends upon the presence of an anti-abscission regulator which is gibberellin. Spraying with auxin puts this

anti-drop regulator out of commission with the result that the fruit falls from the tree. It would seem that the IA inhibition of GA is a general principle of great significance in the process of growth regulation.

We have discussed one aspect of GA/IA interaction: the inhibition of GA-controlled growth mechanisms by IA (or auxins in general). There is another facet to this interaction: the promotion of IA-controlled elongation by GA. This was first shown by Hayashi and Murakami (1954) in the upper part of young oat leaves, in which GA boosted the elongation brought about by IA. Brian et al. (54, 55) reported in pea internode sections that elongation caused by 10 ppm IA can be increased further by as little as 0.01 ppm GA. In our laboratory we demonstrated a similar effect on sections of the classical *Avena* coleoptile. This organ, of course, responds principally to IA, but this elongation effect can be boosted by additional GA.

FURTHER CONSIDERATION ON THE MECHANISM OF CELL GROWTH

Although emphasis in this review is on auxins, it must be understood that it is written in the hope of making a contribution to a deeper understanding of the physiology of growth. For this reason it is significant to realize that as a result of very recent developments, auxin can no longer be recognized as the only plant hormone causing cell elongation. As early as 1954, Hayashi and Murakami showed that the upper part of young oat leaves respond to IA; the lowest parts of these same leaves, however, could not elongate under the influence of IA, but did elongate under the influence of gibberellic acid.

Another observation along the same line is that by Brian and Hemming (1957) who observed that internode sections of peas growing under optimal IA could be made to elongate still further by added GA. This illustrates clearly that GA is a growth regulator which brings about cell elongation but does so by a different route than IA. Similar observations were recently made by Hillman (131).

Phinney et al. (200, 201) have obtained a startling increase in growth of corn dwarfs by application of small amounts of GA. GA brought about elongation which could not be effected by any of the auxins.

Even though GA was originally discovered as a product of a fungus,

there is compelling evidence to consider gibberellins naturally occurring products (hormones) of higher plants (201, 159, 209).

Although it can be argued that GA may only act in the presence of IA, there is evidence that this is not so. Schroeder and Spector (1957) cultured citron pericarp in vitro. As can be expected from a non-tumor tissue, this tissue did not grow unless provided with IA. However, it could be made to grow without IA when GA was provided. As with the pea sections mentioned above, optimum IA effect could be boosted with GA. It would seem therefore that the old axiom, "without auxin no growth" (289), is not as convincing as it once was believed to be. However, Went's original expression, "Ohne Wuchsstoff kein Wachstum," is still valid if one considers the broader term "Wuchsstoff" equivalent to "Growth Regulator" (see definitions, above).

A further point which will be elaborated more fully below when the synthetic auxins are discussed, is the mode of action of auxin. How does it bring about softening of the cell wall? Or, if one accepts the pectin theory, how does it bring about decalcification and subsequent methylation of pectins? We know this phase of growth to be an aerobic, ATP-requiring process. There is no direct relationship between IA and ATP. Skoog (121, 226), who found increased nuclear production in cells as a result of IA treatment, has suggested that the growth regulation mechanism in plants might be located in the nucleus. All efforts to consider auxin as a coenzyme have failed, in plant physiology as well as in animal hormone physiology (126). Peters (1956) has suggested that hormones might act upon the cell as a whole and calls attention to the "fluid anatomy" of the cell which has been referred to by J. Needham as the "cytoskeleton." Mueller (175), discussing steroid hormones, also considers the matrix in which the enzymes lie a logical site for hormone action. An agent which changes the cytoskeleton might be expected to modify several enzymic reactions simultaneously by giving new direction to this fluid anatomy. From the point of view of this working hypothesis, the search for one enzyme reaction which the hormone modifies is doomed to failure. The intimate way hormones act would thus become a problem of surface chemistry. The idea is also quite attractive for plant hormone action, and is discussed below in detail.

BIOCHEMISTRY OF NATURAL AUXINS

INDOLEACETIC ACID AS A MAJOR NATURAL AUXIN

Even though indoleacetic acid was known to chemistry since 1885, it was not recognized as a plant growth substance until 1934 when Kögl and Haagen-Smit identified it as a plant growth-promoting factor from urine (151). At that time it was by no means clear that IA was the native auxin of higher plants; on the contrary, it was generally held that "auxins-a and b" were the auxins of higher plants, while IA was a product of fungi and bacteria. This early belief was based, among other things, on the findings of Went (1928) that the growth substance diffusing from *Avena* coleoptile tips has a molecular weight of 375. This figure was derived from the determination of the diffusion coefficient of the growth regulator. Since Kögl and Haagen-Smit isolated "auxin-a and b" with a MW of 325, and indoleacetic acid with a MW of 175, it only seemed logical at that time to conclude that the *Avena* tip contains "auxin-a or b" rather than IA. However, in 1942, Haagen-Smith et al. isolated crystalline IA from mature maize kernels and again in 1946 (119, 120) from immature kernels.

As early as 1940 an auxin with the diffusion coefficient of IA was extracted from the autotrophic plant *Macrocystis*, a large brown marine alga (257). In 1945 our reseach team in Puerto Rico made it likely that the auxin in sugar cane is IA, not "auxin-a and b" (266), and later Gordon and Sanchez, belonging to the same group, identified IA with considerably certainty as the auxin in pineapple tissue (110).

In 1948 Wildman and Bonner (294) obtained diffusate from 25,000 *Avena* coleoptile tips and subjected it to the Salkowski reaction. They showed that the U V absorption spectrum of the Salkowski derivative was identical with that given by pure IA. From this and other supporting evidence it was concluded that the auxin of *Avena* diffusate was indoleacetic acid. By the end of the last decade it was thus becoming clear that indoleacetic acid is the principal naturally occurring auxin.

At this point one might speculate why Went obtained such a high MW for the auxin diffusing from intact *Avena* coleoptile tips. It was obviously not the result of an experimental error, since similar results were obtained by others. It is now becoming clear that growing tips produce gibberellin as well as auxin (159). It is further a matter of record that growth, which is principally controlled by auxin, is boosted

by gibberellin (55, 125). The *Avena* coleoptile is such a tissue (267). The growth hormones diffusing from an *Avena* coleoptile tip into an agar block can thus be assumed to be a mixture of IA and GA. GA has a much larger MW than IA, and in a diffusion test GA will lag behind IA. Determination of the diffusion coefficient depends upon the relative distribution of the growth-promoting principle in a stack of four agar blocks. It is clear that the top blocks have IA plus GA, while the bottom blocks have only IA. In the coleoptile test GA may be expected to boost IA (see above). Therefore it would appear that a slow moving auxin was being analyzed, while in effect the results were due to a slow moving booster of IA, presumably GA.

Early in the present decade a new technique was developed in auxin physiology which has aided much in the precise identification of naturally occurring growth regulators. This technique is partition chromatography on paper. It affords a ready characterization of indoleacetic acid far more specific than diffusion measurements or tests of stability in acid or alkaline media, because the separations effected are due to partition coefficient or solubility differences and may be checked by a reasonably specific spot color reaction. Auxin paper chromatography was developed first in England (Bennet-Clark, Tambiah and Kefford, 39, 40, 244) and Japan (Yamaki and Nakamura, 1952), and is now used universally wherever auxin has to be identified. While in the past, auxins were identified by curvatures such as those in the *Avena* test, now any serious auxin analysis of plant extracts must involve separation and identification by the chromatographic technique.

The partition solvent most commonly used is still the same as that worked out by Tambiah in 1951: isopropyl aclohol, water, and ammonia in ratios 10:1:1. After the components of the extracts have separated on the paper, it is dried and part of it sprayed with a reagent which locates known compounds. For example, perchloric acid and ferric chloride color the IA spot crimson; Ehrlich reagent colors indole compounds purple; the IA spot fluoresces in UV, etc. A similar part of the paper is cut into squares and dropped into beakers containing coleoptile sections. The amount of growth caused by these squares in these coleoptile sections tells the distribution and potency not only of the growth promoters but also of the growth inhibitors in the chromatogram (Kefford, 40, 143, 144).

In recent years many plants have been extracted and analyzed

chromatographically (65, 186, 228, 246). Indoleacetic acid has almost always been found. Thus the earlier conclusion was firmly established that indoleacetic acid is a major natural auxin in plants.

INDOLEACETALDEHYDE, ETHYL INDOLEACETATE, AND INDOLEACETONITRILE AS AUXIN PRECURSORS

Before the development of the chromatographic technique it had already become clear that materials related to indoleacetic acid exist in plants. The existence of indoleacetaldehyde (VI) in the neutral fraction of plant extracts was made likely by the work of Larsen in 1944 and virtually proven by Gordon and Sanchez in 1949.

Redeman et al. (1951) isolated the ethyl ester (VII) of IA from immature maize kernels. Teubner (1953) demonstrated the same ester in endosperm of apples; although Luckwill and Powell (165) found neither IA nor its ester in apple seeds.

Indoleacetonitrile (VIII) is another neutral material which was obtained in crystalline form from cabbage by Jones, Bentley et al. of the University of Manchester in 1952 (127, 141). Upon alkaline hydrolysis the nitrile yields indoleacetic acid. In some plants, but not all, indoleacetonitrile (IAN) is converted to indoleacetic acid. It is believed that the nitrile as such is inactive and has to be converted to indoleacetic acid in order to be active. The nitrile promotes straight growth of *Avena* coleoptiles but is not reactive on maize, pea and lupine (249) because these plants are poorly capable of converting the nitrile to IA. In the tissues in which IAN is active as an auxin it is more reactive than IA itself. This may be attributed to a better penetration into the plant by the neutral, more fat-soluble material. The above mentioned ethyl indoleacetate is also more active than IA itself for similar reasons.

The Proceedings of the Wye College Symposium on Plant Growth Substances (7) contain many reproductions of chromatograms of extracts of many plants, showing IAN in addition to IA. Examples in which IAN was found: tomato ovary tissue, immature bean seeds, grapes, *Parthenocissus* tissue, apical meristems of *Adianthus*. It was not found in maize, nor in peas, which are plants that cannot hydrolyze IAN to IA.

In higher concentrations, IAN is inhibitory, like IA. With this in mind it has been suggested by Bentley and Bickle (1952) that the

growth inhibitor found in radish cotyledons by Stewart in 1939 might be IAN. This inhibitor was a neutral substance, which was also found in cotyledons of mustard and other crucifers. It could be hydrolyzed to yield an auxin, with properties resembling those of indoleacetic acid. Libbert (1955) found a similar IA-yielding inhibitor in peas.

Not only does IAN act as an auxin precursor and as a growth inhibitor, but it has also been shown to be an activator of indoleacetic acid (Osborne, 1952). Although IAN does not produce a pea test curvature at 20 ppm, its presence in the medium with IA boosts the response of 0.1 ppm of IA from a 2° to a 57° curvature. It is possible that IA is saved from destruction by IAN in an auxin-sparing reaction similar to the one demonstrated by van Raalte (1954) between IA and indole. This auxin-sparing reaction was due to the indole tying up the IA oxidase. In the case of auxin-boosting by IAN, the latter might equally engage the IA oxidase and prevent it from destroying IA. Veldstra (272) is of the opinion that a very large number of synergistic activities are based on a sparing action, that is, competition at a site of loss, not at the site of primary activity. Competition at the site of primary activity always must result in inhibition.

It will be clear, then, that the following indole compounds exist in nature as auxin precursors: indoleacetonitrile and indoleacetaldehyde. There is doubt about ethyl indoleacetate.

BIOSYNTHESIS OF IA FROM TRYPTOPHAN

There is little doubt that tryptophan (I) is the universal and primary precursor to IA. All plants tested so far have enzyme systems capable of producing IA from tryptophan. This was first found in cultures of the mold *Rhizopus,* studied by Thimann in his early attempts to isolate and identify auxin. Indoleacetic acid was produced only when tryptophan was present in the medium. Gordon (106), who is studying indoleacetic acid biosynthesis in detail, has found that the enzyme system is probably non-particulate and occurs as a soluble enzyme in the cytoplasm of leaf cells (107).

The immediate precursor to IA is probably indoleacetaldehyde (VI). It requires an enzyme to oxidize this aldehyde into IA. Such enzymes are found in milk, but also in soil. In Puerto Rico we routinely converted the neutral fraction of pineapple plants, the so-caled bound auxin, into IA by shaking it with soil. The enzyme which converts indoleacetaldehyde to IA has another interesting property. It is ex-

ceedingly sensitive to ionizing radiation. It has long been known that upon radiation with X-rays the auxin content of a plant drops. This was first thought to be due to destruction of auxin per se. The careful researches of Gordon, however, showed that it is not IA which is destroyed but the enzyme which converts indoleacetaldehyde to IA. Upon radiation, indoleacetaldehyde was found to accumulate while IA failed to appear (104, 112).

The steps which lie between tryptophan and indoleacetaldehyde will not be discussed, as they are not clear at present. The conversion apparently is not simply via indolepyruvic acid or via tryptamine or via indoleacetonitrile. Of course it is quite possible, even likely, that the conversion does not follow a single pathway, but that alternatives exist in nature. Recently the unexpected discovery was made by Paleg and Gordon (1956) that phenolases, such as tyrosinase, activate the oxidation of tryptophan to indoleacetic acid. It was even found that tyrosinase, catechol and tryptophan without any other enzymes produced high yields of IA. It is obvious that this field is in a state of flux at this time, and it is therefore difficult to draw any definite conclusion outside of the well established fact that tryptophan is the primary precursor to the natural auxin, indoleacetic acid. Tryptophan is not necessarily converted to free auxin, since recently Good and Andreae (1956) showed that tryptophan conversion did not stop at IA but continued to a conjugate of IA with aspartic acid. In this way IA may be more stable against destruction by the IA oxidase (29, 102).

Using a micromethod, it was found that cotyledons and the apical meristem of seedlings were richer in free tryptophan than other parts of the plant (188). Tryptophan is formed from indole and serine (113, 181). Zinc is required directly for the synthesis of tryptophan and thereby indirectly for the synthesis of indoleacetic acid. The interrelation between zinc and auxin was recognized as early as 1940 by Skoog (225).

FATE OF IA IN THE PLANT

Although we know of a number of reactions involving IA in the plant, we do not have a clear understanding as to which reaction leads to growth. As noted earlier, this may not be a biochemical reaction at all, but some physical-chemical effect on the cystoskeleton of the cell.

Galston et al. (93) have found that when pea seedling tissue is

treated with IA, some of the IA (not over 10%) ends up by being bound to a protein. All pea tissue will show this reaction, even if it is no longer capable of growth. The binding of IA to protein is an energy-requiring aerobic process. The resulting bond is a strong one and even boiling will not release the IA from the protein. However, co-enzyme A will break up the complex, but not into its original components. The resulting auxin is not free but may be an IA peptide. What the physiological significance of this reaction is, we do not know. One attractive suggestion was made by Galston at the Wye College Conference in 1955 when he commented that the IA protein might act as a sequestering agent to protect IA from its oxidase. It is well known that when IA is introduced into tissue sections, much of it is destroyed by IA oxidase within a short time. One often wonders how any free IA can escape this destructive action. Protecting it by association with protein may well be an answer. On the other hand, it is possible that the IA oxidase acts only when released by wounded cells (49).

The IA oxidase was isolated in 1947 by Tang and Bonner from pea tissue, but has been found in many other plants since that time. It is an oxygen transferase (170). In IA oxidation one mole of O_2 is absorbed and one mole of CO_2 given off per mole of IA inactivated. This prompted the belief that indolealdehyde (IX), not indoleacetaldehyde (VI) which we discussed earlier as an IA precursor, was formed as the inactivation product. There has been much argument against this view. However, the aldehyde is certainly one of the reaction products of IA oxidase. Racusen (1955) in our laboratory, and recently Stutz (1956) in Gordon's laboratory, have confirmed the early conclusion by Bonner (9) that indolealdehyde is formed. Ray and Thimann (1955) and Manning and Galston (1955), however, have evidence for other breakdown products. Several other types of IA oxidases have been shown to occur in different plants (Briggs et al. 1955) with properties which differ from that of the original pea IA oxidase (216, 252). It is possible that the breakdown of IA in the plant follows along several pathways. W. D. Bonner in a recent review (1957) also denies the existence of one specific IA oxidase.

The activity of IA oxidase in the plant is the reverse of that of its IA content, as is reasonable to expect. Thus in roots which have a low IA content, the IA oxidase activity is high. In the growing

regions of the shoot, which obviously have a high auxin level, the IA oxidase activity is low. In the older regions of the shoot the IA oxidase activity increases progressively (202). Recent evidence (94) indicates that an inhibitor of IA oxidase occurs in the plant which appears to be the controlling factor in the oxidase activity.

If young tissues, low in IA oxidase, are treated with synthetic IA, their capacity to destroy IA increases. It thus appears that we are dealing here with induced enzyme formation (93, 95). Maximum induction for the pea was found at 10^{-7}M (about .2 ppm) IA. It is interesting to note here that the presence of IA in the young cell not only initiates elongation but also sets in motion the mechanism for its own destruction.

The IA oxidase is a complex system which, according to Galston et al., is composed of two components. The oxidation of IA is achieved by a peroxidase. The H_2O_2 required for the oxidation of IA by peroxidase is produced by a peroxide-generating enzyme which might be a flavoprotein. Kenten denies the need for a flavoprotein in the oxidation of IA (146).

It has been shown that H_2O_2 is not limiting in the reaction, but the peroxidase is. It further turned out that the peroxidase is the adaptive member in the system (Galston and Siegel, 1954). It is the peroxidase activity which goes up after IA treatment. This peroxidase activity does not increase equally in all tissues treated with IA. Jensen (140) found that in the root it is especially the young vascular tissue (protoxylem and phloem) which responds.

This has very intriguing consequences. These young provascular cells are the potentially lignified cells of the vascular system. IA has been shown to induce lignification in pea roots and formation of xylem strands in wounded stems (Jacobs, 136-139). Siegel (1955) has shown that peroxidase, together with peroxide and a lignin precursor such as eugenol, causes lignin formation in plant cells. For instance: one can take a cotton fiber, dip it in pea peroxidase, apply eugenol and H_2O_2, and the fiber will become coated with lignin. It was shown indeed (Jensen) that those cells which are induced by IA to higher peroxidase activity have a greatly increased ability to convert eugenol to lignin.

Again we have an example here of the far reaching consequences of the effects of IA on plant tissue. Earlier we discussed the effect of

IA on the wall enzymes. Later we will see many more manifestations of auxins. Many of these are often especially strong with synthetic auxins.

Finally, the destruction of IA in plant tissues by light must be mentioned. Goldacre (1954) isolated a protein-free material by dialysing crushed pea tissue. This material sensitizes the destruction of IA in light, especially in the blue-violet. The inactivation of IA probably involves an oxidation without intermediate formation of H_2O_2. Riboflavin is the best sensitizer for the destruction of IA solution in vitro by visible radiation (Brauner, 1953), but there is no evidence that it has a function in the growth inhibition by light (Mer, 1957). Which photoreceptor pigment in plants brings about retardation of growth and phototropic curvatures is not clear at present. Carotene and riboflavin have been suggested, and a system involving several pigments is likely.

SYNTHETIC AUXINS

The only auxins so far discussed have been indole compounds. Today literally hundreds of synthetic compounds with auxin activity are known which are not indole compounds (87, 141a, 213). These will not be reviewed extensively, however, as this has been done admirably by Veldstra (26, 273). Instead, a few synthetic auxins will be discussed which I consider critical for understanding the reasons why some synthetic compounds possess auxin activity, while some very closely related chemicals appear completely inactive.

Most of the synthetic chemicals showing auxin activity do so at a low level. A few, however, surpass native auxin in activity, and nearly all persist in the plant much longer than native auxin. As we will see later, it has been the few compounds with a combination of high activity and high persistence that have been most useful in agriculture.

The simplest synthetic auxin is perhaps phenylacetic acid (X). It has 1 to 10% of the activity of indoleacetic acid (II), depending upon the test used for evaluation (8). When the molecules of phenylacetic acid (PA) and indoleacetic acid (IA) are compared, one notices (*a*) that they are both acids, (*b*) that both have aromatic rings, and (*c*) that the acid group can assume a position outside the plane of the ring. In order to observe the latter it is advantageous to make the molecules with Fisher atom models. It has been found that all auxins of

high activity have these three features in common. These are rather loose requirements, and for the relatively weak auxins the requirements for activity are less strict yet. For instance, in recent years a dithiocarbamate (225, 271, Table II) has become known with an auxin activity of 1% of that of IA. It has an acetic acid group but does not have a ring. One must assume that the function of the ring is taken over by another arrangement of atoms.

In spite of this apparent lack of strict requirements for auxin activity, seemingly small changes in the molecule may render it inactive. Consider phenoxyacetic acid (XI). It is inactive, yet it differs from phenylacetic acid only by one single oxygen atom. This inactivity of the phenoxyacetic acid (POA) structure is not inherent to the phenoxyacetic acids as a group, however. Addition of a methyl group to the alpha carbon of the side chain (XII) restores the activity to the level of phenylacetic acid. Addition of a chlorine atom to the ring (XIII) bring the level of activity up to that of IA. And, addition of two chlorine atoms (V) increases the auxin activity of phenoxyacetic acid so much that it surpasses that of IA by a factor of 10. Compound (V) is 2,4-dichlorophenoxyacetic acid, the famous 2,4-D which is used annually in millions of pounds as a selective herbicide in cereal crops.

WHY PHENYLACETIC ACID IS ACTIVE AND PHENOXYACETIC ACID IS NOT

One obvious difference between the two molecules is that the ratio between hydrophilic (polar) groups and lipophilic groups is more in favor of the lipophilic properties in PA than in POA. PA will thus partition into fatty phases in the cell more readily than POA. Veldstra (274) has pointed out that active auxins have a definite hydrophilic/lipophilic balance. One might add that many biologically active substances have a definite hydrophilic/lipophilic balance in their molecules and that within a given series the more active materials have this balance in favor of lipophilic properties.

The reason for this is that, even though we do not know exactly where in the cell an auxin molecule has to go in order to set off a growth reaction, it is nevertheless a reasonable assumption that auxins have to pass through fatty phases and/or accumulate (solubilize) into these. The cuticle, the plasma membranes and mitochondria are all cell parts rich in fat and which may well be phases through which

auxins pass or into which they solubilize. The partitioning properties between a water and an oil phase have been determined for both PA and POA (263). It was found that when these acids are shaken in a mixture of water and glyceroltrioleate, three parts of PA go in the oil for every four parts that go in the water. For POA acid, only 1.6 parts go in the oil for every four parts that go into the water. In other words, the affinity for fat of PA possessing auxin activity is nearly double that of POA, lacking auxin activity.

There is one further difference between phenyl and phenoxyacetic acids which has to do with the frequency with which the carboxyl group can assume a position outside the plane of the ring. This difference is more obvious between *cis*- and *trans*-cinnamic acids than between phenyl and phenoxyacetic acids. For this reason the cinamic acids will be discussed first. *Trans*-cinnamic acid (XIV) is inactive as an auxin. On Fisher atom models it can be seen that the side chain of the *trans* form can rotate relative to the ring in such a fashion that positions of the side chain in the plane of the ring are favored. In the *cis* form (XV), on the other hand, which is active as an auxin, rotation of the side chain favors much more a position of the carboxyl group outside the plane of the ring. This difference in spacial arrangement of the cinnamic acids has been recognized for over two decades. It has been further found in many other compounds (26) and has now been so definitely proven that the third general rule for auxin activity can be considered well established: the acid group must be so arranged that its most frequent position is outside the plane of the ring.

We can now return to compare phenyl and phenoxyacetic acids. On Fisher models it can be observed that the angle of the axis of the acetyl group (through the carbons) with the axis of the ring (through carbons 1 and 4) is such in phenoxyacetic acid that intramolecular rotation causes the side chain to be in the plane of the ring most of the time. This condition is incompatible with the third rule of auxin activity. By contrast, the rotation of the side chain relative to the ring in phenylacetic acid is such that the carboxyl group is likely to be more often outside than in the plane of the ring. This condition is compatible with the third rule for auxin activity.

After this analysis it is relatively easy to understand why α-methylphenoxyacetic acid (XII) is active. The methyl group (*a*) shifts the

hydrophilic/lipophilic balance in the direction of greater fat solubility, and (*b*) restricts the rotation of the side chain so that the carboxyl group will be outside the plane of the ring more often than in phenoxyacetic acid itself.

FURTHER STERIC FACTORS, VAN DER WAAL'S FORCES AND HYDRATION

The increased activity of chloro (XIII) and dichlorophenoxyacetic acid (V) over phenoxyacetic acid itself (XI) is probably connected again with a more favorable balance between lipophilic and hydrophilic constituents of the molecule (26). Since it makes much difference in the auxin activity of the molecule in which position in the ring the chlorine is introduced, it is obvious that there are position as well as partition effects. Partition is probably important in getting the auxin molecule to the site of action, while position effects (steric effects) are important at the ultimate site of action.

The question of the first step in any biological reaction initiated by active biological materials is an interesting one. Gunther, Metcalf et al. (115) have considered it for insecticide activity. Mullens has considered it for insecticide action (180) as well as for auxin activity (unpublished). We will follow a similar line of reasoning.

Even though we do not know how auxin brings about its physiological effects, it is most likely that the first step must involve penetration of the auxin molecule into something. This something may be a protein, or a coloidal micelle, lipoprotein, and probably will be part of the cytoskeleton, or enzyme matrix. In order for the auxin molecule to be effective, it is not enough for it to penetrate into an intermolecular cavity; it must remain there. The auxin molecule gets into the cavity by thermal agitation (a two-step process), but it must not be thrown out again by this same action. It is the Van der Waals forces which hold the auxin molecule in the cavity.

Let us suppose that we are dealing with a phenoxyacetic acid and that the molecule penetrates the cavity ring first. The 4 and 5 positions on the ring then constitute the head, and the groupings on the 2 and 1 positions the tail of the auxin molecule. One can visualize the cavity as a 3-dimensional slot and the auxin ring as a coin. It will penetrate only if it fits the slot. Suppose the slot measures 6.5x3.5A. 2,4-D, 2,5-D and 2,4,5-T will just pass through it (Fig. 1). However, all the 2,6 substituted compounds are too wide to fit the slot: 8.5 A mini-

mum diameter (Fig. 1). Consequently 2,6-D (XX) is inactive (141a, 155). It penetrates only very superficially and is thus easily dislodged. The 2,4 and 2,5 compounds, by contrast, penetrate deeply, and the groups on the 4 and 5 positions will penetrate deepest. When there

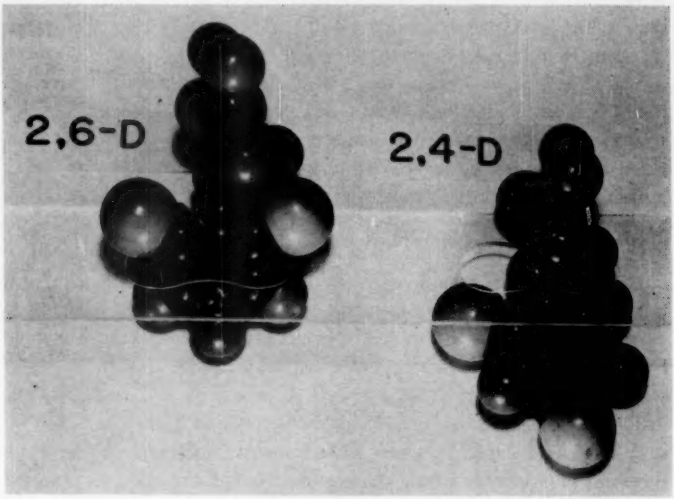


Figure 1. Difference in degree of penetration of models of molecules of 2,4-D and 2,6-D in a slot having maximum dimensions of 6.5x 3.5 cm (corresponding to 6.5 x 3.5 A). The sheet of plastic which contains the slots is imagined to represent a portion of the cytoskeleton. While the highly active auxin 2,4-D has penetrated deeply and is well anchored, the nearly inactive auxin 2,6-D can penetrate only superficially and thus is easily dislodged from the surface. Another important consequence of this shallow penetration of the 2,6-dichlorophenoxyacetic acid molecule is that its side chain protrudes too far beyond the surface. This prevents the carboxyl group from carrying out the postulated primary auxin reaction: acting like a "knife switch" to activate the H-bond network at the surface of the cytoskeleton.

is only hydrogen on these groups the hydrogen atoms will not contribute much to the anchoring of the auxin molecule inside the cavity, as the Van der Waals forces are proportional to the atomic weight. By contrast, when heavy groups such as chlorine are in the 4 and/or 5 position, they serve to anchor the auxin molecule tightly inside the cavity. It is not surprising, therefore, that the compounds possessing these effective anchoring groups have the highest activity among the

chlorophenoxyacetic acids: 2,4-D, 2,5-D and 2,4,5-T (155, 281). See ADDENDA for further discussion.

One might next ask, what could an auxin molecule accomplish when it is sorbed, say, to the cytoskeleton. If one assumes that this structure is for a large part protein, one is immediately confronted with problems of hydration. According to Bull (61), there is no water inside the protein molecule as such, but there is a large volume of water surrounding the molecule—some 28% is mentioned as an average figure (p. 321). Recently Klotz and Ayers (1957) also concluded that protein molecules have a water envelop in which the water molecules are arranged as in ice. Such an orderly arrangement of water molecules around protein molecules may be limited to local regions of the surface or may be general around the whole protein molecule. They point out that "When a molecule, e.g., O_2 (or in our case, an auxin molecule) combines with the prosthetic group or specific site, the structure of water at this point could be changed significantly ... it is evident that the effects due to combination at a specific site could be transmitted some distance and result in a disordering of the water structure some distance away". Such an effect by the auxin on the hydration of individual protein molecules would therefore result in a change in distance between these protein molecules in the cytoskeleton. This in turn would change the relative distances between the enzyme molecules attached to this cytoskeleton. This again would mean a change in the rates of reaction between these enzymes, resulting in the production of specific metabolites at changed rates. The end result would be a changed ratio between metabolites in the cell. Skoog's researches (see above) have shown that it is just such ratios which are responsible for the physiological pattern of a tissue.

One might ask, how could relatively few auxin molecules have such a drastic effect on the hydration of the enzyme matrix. This again may be visualized by another example of hydrogen bond chemistry. Huggins (1957) has pointed out that the strength of hydrogen bonds can be increased when hydrogen bonds are arranged in rings or chains connected through an oscillating system of atoms. Suppose the acid group of an auxin would be a link in such a system. It could act like a switch in a relay system. (See ADDENDA, Fig. 2).

Hydration may well be a fundamental mechanism in many physiological reactions. Thus Szent-Györgyi (1956) states: "It may be that

muscular contraction, essentially, is a rearrangement of protomyosins, consecutive to a destruction of their surrounding water structures, while relaxation is the reestablishment of this hydrate envelope". In the animal hormone field it has been shown that thyroxin causes changes in the structure of isolated mitochondria, leading to uptake of water and swelling (156). First, among the physiological effects of estrogens is a drastic increase of water content (175).

It is a fascinating thought that the action of the plant hormone, auxin, might start with the hydration of a few protein molecules and end via a long chain of enzymatic events in the enormous osmotic water uptake of the whole cell which characterizes elongation.

THE TWO-POINT ORTHO-REACTION HYPOTHESIS

In the above discussion we have considered the first step of the auxin reaction involving only H-bonds, Van der Waals forces, steric hindrance and other physical chemical factors. There is another school of thought, however, which considers that the first step in the auxin reaction is a chemical reaction between auxin and substrate on which it acts inside the cell. Went and Thimann (1937: 136) expressed this view, and in his second review of this series (1945: 490) Went noted that the view that auxin is a coenzyme had gained ground. This concept of auxin being bound by chemical (covalent) bonding to a substrate reached its peak in the Two-Point Ortho-Reaction Hypothesis of Muir and Hansch (1951, 1953). This theory proposes that an auxin molecule reacts with some entity in the plant at two points: through the carboxyl group, and through the carbon in the ring ortho to the side chain. Only when these two bonds are made is the auxin capable of exerting its physiological effects. In their 1953 publication (179) an example is given in which the cysteine side chain of a protein is the site for the two-point bonding with 2,4-dichlorophenoxyacetic acid. The carboxyl group of the auxin forms a peptide bond with the amino group of the cysteine, and the *ortho* carbon of the auxin forms a C-S-C bond with the sulfhydryl group of the cysteine group of the protein. This theory was based on the observation by Muir et al. that whenever the two positions *ortho* to the side chain were substituted, phenoxyacetic acids were without auxin activity. This theory became very popular for a while, as it is rather precise and logical and seemed to fit the facts. However, at present there is so much evidence against it, that the Two-Point

Theory, as formulated by Muir and Hansch, must be considered untenable. The first difficulty arose when Bentley (1950) found that di*ortho*substituted *benzoic* acids (XVI) have a high auxin activity. In fact, these materials require a chlorine, or substitution of similar bulk, on both *ortho* positions. The proponents of the two-point theory then argued that chlorine on the benzene ring next to a carboxyl group renders the *ortho* position especially reactive; just the reverse of what was argued with the phenoxyacetic acids. However, Veldstra (273) proved that 2,3,6-trimethyl substituted benzoic acid (XVII) was active, as is the 2,3,6-trichloro substituted benzoic acid (XVI) which made the argument of reactive *ortho* positions untenable for the benzoic acids. The function of the bulky 2,6- substitutions in the benzoic acids is undoubtedly a steric one, such as forcing the carboxyl group outside the plane of the ring—the third requirement for auxin activity! (See ADDENDA).

In the meantime it had become known that the amide of 2,6-dichlorophenoxyacetic acid (XVIII) is perfectly active as an auxin, while the acid itself (XX) is not, as we have seen. Finally, Osborne et al. (1955) showed that the introduction of a methyl or ethyl group on the alpha carbon of the side chain of 2,6-dichlorophenoxyacetic acid produced activity (XIX). Clearly, then, we have here a case similar to that discussed above with phenoxyacetic acid (XI), and alpha methyl phenoxyacetic acid (XII), showing that inherently the 2,6-dichlorophenoxyacetic acids are capable of inducing an auxin response.

The most conclusive recent evidence against the need for a free *ortho* position for the auxin activity of the phenoxyacetic acids came from J. Bonner (private communication). He showed that 2,4-dichloro-6-fluorophenoxyacetic acid has good auxin activity. There was no evidence of the splitting off of F to free the *ortho* position.

It is our conclusion that the most satisfactory explanation of the low activity of an auxins having two bulky groups on the two *ortho* positons is one of steric hindrance: the ring becomes too wide to fit the cavity (slot) in the cytoskeleton. This same simple view also explains the "anti-auxin" activity of these 2,6 substituted acids. They block the slot part of the time, thereby reducing the rate of penetration of the active auxins.

This brings us to a consideration of what happens to the acid group. Again, here it is not necessary to assume that the carboxyl

group will react through a covalent bond with a substrate. Is is perfectly possible that the change in pattern of surface charges, brought about by the carboxyl groups of the auxin molecules sticking out of the surface (Fig. 1), is sufficient to change the pattern of interaction between enzymes on the cytoskeleton or enzyme matrix (see above). This in turn would change the pattern of biochemical reactions and result in the physiological responses of auxin characteristic for a specific tissue. The auxin molecule need not participate at all directly in the chain of biochemical events that it sets off. One might imagine that a number of physiological relays are involved in the system. An exceedingly small cause can have large results. A mosquito can change the course of a mighty fleet.

Closely associated with the Two Point Theory is a theoretical treatment by Bonner's group, known as the "chemical kinetics of growth" (46). This treatment is identical to enzyme kinetics but does not imply that auxin-induced growth is enzymatic (47). Further, it is quite independent of whether the ring reactive position is the *ortho* position or whether it is just the geometry of the whole ring (J. Bonner, private communications). The reviewer is of the opinion that if the auxin reaction could be studied in a cell-free medium, such considerations would be more valid. This is not yet possible. Consider, for example, the V max. This is the maximum growth rate which is assumed to be achieved when the auxin-receptor sites in the cell are fully occupied by auxin molecules. The starting point of all considerations is the growth-auxin concentration curve of coleoptile sections growing in buffered sugar solutions containing auxin. In such a curve growth first increases with auxin concentration, but soon reaches a maximum where further increases in auxin concentration no longer produce additional growth. The simple assumption that this maximum growth is reached because the cell runs out of auxin-receptor sites is probably not true. The plant most likely runs out of other growth factors as the following will show.

Growth curves such as we are considering here have been known in plant physiology as "Blackman curves". Went (1935) considers the growth-auxin concentration curve "one of the finest examples of Blackman's theory of limiting factors". In the complex process of growth, auxin saturation is reached and another factor becomes limiting. Growth stops when another growth factor runs out and not neces-

sarily when all auxin receptor sites have been occupied. That this is so can be seen from Bonner's own work (1949). In an article entitled "Limiting Factors and Growth Inhibitors in the Growth of the Avena Coleoptile" he shows that the maximum growth of sections growing with optimal sucrose and IA in the medium can be increased 50% by the addition of 100 ppm of arginine, and 75% by the addition of arginine and 100 ppm manganese sulfate! In the presence of adequate auxin, carbohydrate, arginine and manganese, growth may be still further increased by addition of thiamin or nicotinic acid. The maximum growth rate of coleoptile sections growing in auxin—containing sugar solutions (upon which the theoretical framework rests) is therefore obviously not due to a lack of auxin-binding sites.

An example of the dubious value of theoretical deductions based on the Two Point Theory is the explanation of the inhibition of growth by high concentrations of auxin: "If the growth functional form of auxin consists of a two-point attached auxin-receptor complex, then we must anticipate that at a sufficiently high auxin concentration, two molecules of auxin will simultaneously bind to the receptor entity, each remaining bound through but a single attachment point" (47). Growth takes place, according to the Two Point Theory, if one single auxin molecule attaches to both attachment points. Therefore, "inhibition of growth by high auxin concentration would appear to be a natural and indeed an inescapable consequence of two-point binding of auxin to receptor at lower auxin concentrations" (47). However, there are much more obvious reasons why high auxin concentrations inhibit growth. Bennet-Clark (38) has made it likely that it is due to progressive toxicity. This is easy to understand as the following consideration will show. The tonoplast, the membrane responsible for the semi-permeability of the cell, is usually considered to be composed of a bimolecular layer of fatty substances. Such a structure is a form of colloidal micelle, and materials such as auxins, which are part hydrophilic, part lipophilic (amphiphilic molecules), penetrate into such a structure (solubilization). Since the water-holding properties of the tonoplast are due to the close packing of the lipophilic parts of the molecules of the membrane (palisade arrangement), any penetration (solubilization) of foreign matter into this structure will reduce its water-holding capacity and thus increase its permeability. The more auxin molecules solubilize into the tonoplast, the more its

permeability will be increased. Finally a point is reached where the cell collapses because it cannot hold water osmotically any more. Discussions of permeability changes in the plasma membrane by solubilizing molecules have been presented elsewhere (262, 25). Incidentally, the above considerations also make it understandable why reports of increased permeability of cells due to auxins continue to appear in the literature. It would seem that such an effect *must* be found when unphysiologically high auxin concentrations are used.

THE CARBAMATE AUXINS

This discussion of synthetic auxins was started with the simplest of auxins: phenylacetic acid, and will end with a seemingly most aberrant auxin: S-(carboxymethyl-dimethyl dithiocarbamate) (XXI) (Table II). It was discovered in Holland in a program on systemic fungicides

TABLE II

AUXIN ACTIVITY OF DITHIOCARBAMATES

(After van der Kerk, van Raalte et al., 1955)

$(R_1)_2N-\underset{\underset{S}{\|}}{C}-S-\underset{\underset{R_2}{\|}}{\overset{\overset{R_3}{\|}}{C}}-COOH$		Auxin Activity (Elongation)
$R_1 = CH_3$ $R_2 = R_3 = H$	(XXI)	Active
$R_1 = C_2H_5$ or higher alkyl	(XXII)	Slightly active or inactive
$R_1 = CH_3$ $R_2 = CH_3$ $R_3 = H$	(XXIII)	Active
$R_1 = CH_3$ $R_2 = C_2H_5$ $R_3 = H$	(XXIV)	Active
$R_1 = CH_3$ $R_2 = R_3 = CH_3$	(XXV)	Inactive
$(CH_3)_2N-\underset{\underset{O}{\|}}{C}-S-CH_2COOH$		Slightly active or inactive
$(CH_3)_2N-\underset{\underset{S}{\|}}{C}-O-CH_2COOH$		Active

(van Raalte, van der Kerk, et al, 255 271). That this material is a true auxin, having an activity of about 1% of that of indoleacetic acid, has been confirmed by at least three independent researchers: Veldstra, 273; Gordon et al, 109; Fawcett et al, 88). On paper the structural formula appears to differ much from that of phenylacetic or phenoxyacetic acid. Yet when Fisher models are used to construct the

molecules, it appears that in general outline and configuration, the ring compounds and the carbamate auxins are nearly identical. The dimethyl dithiocarbamate group covers an area identical to the phenyl group, as indicated in Table 1B. Resonance makes both structures planar. The dimethyl dithiocarbamate group also fits the "slot" into which the ring of molecules such as 2,4-D fit (Fig. 1). It will be seen from this that the two methyl groups on the nitrogen serve as the anchoring groups. Each of these groups has a volume equal to that of a chlorine atom, but its Van der Waals binding forces are less than half of those of chlorine.

From a strictly chemical point of view these two groups can hardly be considered similar. Yet from a surface chemical standpoint they may well be similar in function. An analogous case is the following. Veldstra (273) replaced the carboxyl group of indoleacetic acid and of naphthaleneacetic acid with the sulphonic or phosphonic group. He retained auxin activity. Here again, although the sulphonic and phosphonic groups are certainly not equivalent to carboxyl in a chemical sense, these groups are, nevertheless, substitutes (but poor ones) for the carboxyl as far as the physiological functions of the molecule is concerned. From the point of view of surface chemistry, all three groups are polar and, as such, equivalent to a certain degree.

The carbamate auxin has further points of similarity with phenylacetic or phenoxyacetic acid. It has been shown that one methyl or one ethyl group on the second carbon of the acetyl side chain does not impair the auxin activity of phenylacetic or phenoxyacetic acid. Two such groups on this carbon atom abolish activity, probably because of steric hindrance. Similarly in the carbamate auxin (Table II) one methyl (XXIII) or one ethyl group (XXIV) on the second carbon atom does not interfere with activity, but two methyl groups on this carbon atom (XXV) abolish activity. It is clear, therefore, that the function of the side chain in the carbamate auxins is the same as in the phenyl— and phenoxyacetic acids.

AUXIN PHYSIOLOGY AND ITS APPLICATIONS IN AGRICULTURE

THE TRIGGER ACTION OF AUXINS

Even though the identification of auxins was based on the property to promote cell elongation in seedlings, it soon was realized that auxins

affect a multitude of other physiological processes as well. The same concentration of indoleacetic acid (II) which promotes growth of the coleoptile of *Avena*, will inhibit growth of its roots. The same concentration of naphthaleneacetic acid (IV) which promotes growth of the petiole of tomato, will also provide a stimulus for the continued growth and development of the fruit, even in the absence of pollination. Relatively high concentrations of either indoleacetic acid or synthetic auxins will keep lateral buds in dormant condition and will induce root formation on cuttings of many plants. When dropped in the heart of a vegetative pineapple plant, as little as 0.25 mg of naphthaleneacetic acid will make the plant flower and produce a normal fruit out of season.

In all these reactions the auxin acts as a typical trigger. It sets off reactions which are all out of proportion to the original amount of matter put into it. In the case of the pineapple, a 0.25 mg "shot" of auxin results in 2.5 kg fruit. On the weight basis the effect weighs ten million times as much as the cause. One could liken the action of auxin to a coin in a "slot machine." If the coin is dropped in a Coca-Cola-vending machine, a bottle of Coca-Cola comes out; if it is dropped in a coffee machine, a cup of coffee comes out; when dropped in a "juke box", the thing makes noise for a few minutes. The coin is the same in all cases—it triggers something which is poised to go.

What the initial trigger reaction is which causes auxin to set off a chain of events is not known at present. It is likely that it is a physical-chemical event. It is possible that it changes the enzyme matrix by changing its hydration, thereby changing the relation between enzymes. Auxin itself probably does not participate in these enzyme reactions. As a result of all of this, the balance between biochemical reactions undergoes a shift. Thus, recently Reinhold and Powell (1956) analyzed the changes brought about by indoleacetic acid in sunflower tissue that was placed in well aerated solutions of radioactive amino acids. Within 30 minutes after the auxin was applied, changes in the metabolism of the tissue could be observed. After two hours the amount of glutamic acid taken up was increased 72% over the control as a result of the presence of three ppm of indoleacetic acid. Water uptake was increased 10% over the controls. The amount of glutamic acid respired was increased 52% over that of the control. Another

example is in the work of Newcomb (1951) who showed in tobacco pith tissue that under influence of auxins the ascorbic acid oxidase activity increases many-fold. This must mean that inside the cells, at least locally, there is a shift from a more reducing to a more oxidizing milieu. Such a shift must suppress certain reactions, favor others, thus setting up a chain of events which ultimately expresses itself as a physiological response. Cell walls become more plastic, cambium cells divide, root initials develop in the pericycle, in the pineapple the growing point stops producing leaf primordia and shifts to laying down flower primordia, etc.

FLOWER INDUCTION IN THE PINEAPPLE

First we take up the effects of auxin on fruiting. With Nitsch (21) we will consider the fruit to start its cycle with the flower primordium and will therefore discuss how auxins act in these early stages. The only clear cut case in which auxins induce initiation of flower primordia is in pineapple. This was first found by Clark and Kerns in Hawaii. It is known that a variety of auxins, including indoleacetic acid, will set off the flowering reaction in all varieties of pineapple that have been investigated. It is further known that the pineapple has an active auxin metabolism (110, 111). It produces indoleacetic acid from tryptophan (I) with indoleacetaldehyde (VI) as an intermediate product. It destroys indoleacetic acid according to a pattern found in other plants. A high free auxin content is found in the stem apex of the plant, a high "bound auxin" content in the base of the young leaves attached to this stem apex (268). It would appear that these auxin precursors move from the leaf base to the stem apex where they are activated into auxins.

Although efforts have been made to find an increase in auxin level prior to flowering, none has been found. Even though the pineapple has an active auxin metabolism, and even though auxin applied to the vegetative plant will make it flower, it does not follow that flowering of the pineapple plant is necessarily auxin-controlled. For instance, unsaturated hydrocarbons (ethylene, acetylene) caused flower formation of pineapple. Cool nights set off the flowering reaction, which is the reason that natural flowering takes place during winter (265). It is not at all necessary that these two ways of inducing flowers in the pineapple go via auxin. It is not at all unlikely that auxin, ethylene, cold, independently are capable of setting off the reactions which re-

sult in flowering. If one views the action of auxin as a physical-chemical process, it is easier to understand why physical factors, such as cold, can lead to similar physiological end results. It is, of course, entirely possible that a hormone is involved in the flowering process, but this hormone may not be auxin.

Whether or not auxin is immediately involved in the natural process of flowering is immaterial as far as its practical applicability is concerned. Agricultural labor has become increasingly costly. For this reason a high degree of efficiency is required in the production of crops. While in the past, a field had to be picked over repeatedly in the course of a season, at present the whole field can be harvested in one single operation. This has become possible through artificial flower induction. The whole field is sprayed with the flower-inducing agent, all plants in this field produce flowers at the same time, so fruits are all ready for harvest at the same time. In addition, many plants which fail to set fruit under natural conditions will produce when artificially induced. The per acre yield of a field is thus increased as a result of artificial flower induction. Timing is another significant advantage. One can plan it so that one field is ready for harvest after another has been picked. This means that a small picking crew can be kept busy and also that a peak load at the cannery is avoided. Thousands of acres of pineapple are artificially induced to flower each year. Either acetylene (1 gram of calcium carbide per plant) or naphthaleneacetic acid (25 grams per acre) is used, whichever is more practical or economical under prevailing conditions. A summary of auxin physiology in the pineapple may be found in van Overbeek (10, 260) and Leopold (2).

A characteristic of auxins is that, if they promote a process at low concentrations, they almost certainly will inhibit it at high concentrations. Concentrations ten or more times those required to induce flowering in the pineapple will inhibit flowering during the natural flowering season. Auxins are not used in practice for flower prevention of pineapple plants because of undesirable side effects. In vegetable crops, however, considerable progress has been made in controlling untimely flowering (Wittwer, 1954). Alpha-*ortho*-chlorophenoxypropionic acid (XXVI) at a concentration of 100 ppm in a water spray has significantly delayed flowering in both celery and cabbage. For effective results it is essential that the chemical be sprayed on the plants dur-

ing the time that they are subjected to the cold temperatures which induce flowering.

FRUIT SETTING

The involvement of auxin as a limiting factor is more convincing in fruit setting and fruit development than it is in flower initiation. In the normal course of events, it is fertilization that provides the greatest stimulus which causes the ovary, and often surrounding tissue, to continue growth, ultimately resulting in a mature fruit. Auxins are involved in this growth process. First, pollination proper results in the appearance of a "gush" of auxin in the flower and often even in the flower stalk. Secondly, after fertilization, the developing seed becomes a center of auxin production.

Pollen has been known to contain auxin since the early days of auxin investigations (Went and Thimann, 1937). The occurrence of growth-promoting chemicals in pollen was known to Fitting, who in 1909 extracted a growth-promoting principle from *Hibiscus* pollen which stimulated the development of orchid ovaries. Fitting also was the one to use the word "hormone" first in plant physiology.

In 1936 Gustafson showed for the first time that fruits can be produced artificially by treating the flowers with synthetic auxins, bypassing pollination entirely. Normal sized fruits of tomato, bell pepper and eggplant were obtained. These fruits were seedless. Hence auxins can bring about parthenocarpic fruit development. However, in a capable review, Luckwill has pointed out that in many cases auxins fail to induce parthenocarpy in unpollinated flowers (1957).

Gustafson (1939) reasoned that the auxin from the pollen grain and pollen tube might be responsible for the early stages of fruit growth. However, in 1941 the author and his associates (264) realized that the small amount of pollen necessary to pollinate a flower does not carry enough auxin to account for early fruit development. It was thus proposed that pollination releases auxin previously present as a precursor in the female parts. This view was substantiated by Muir (1942) whose measurements showed that over 100 times more auxin is formed in the ovary than is carried to the ovary by the pollen. Recently, Lund (166, 167) further confirmed this view. Enzymatic production of indoleacetic acid in the ovary, probably from precursors such as tryptophan and indoleacetaldehyde, is activated by pollination.

In the course of his pioneer work on auxins in fruits, Gustafson

(1939) determined the distribution of auxin in the growing fruit. Invariably the highest content was in the seeds. It thus appears that the auxin originating from the seed is the controlling factor for the growth of the fruit. Many older observations now become meaningful. In apples and pears, destruction of the seeds in the young fruitlets sometimes occurs as a result of late frost. When this happens, the fruit tissues, although they are not themselves damaged by the low temperature, immediately cease growth and the fruit eventually drops from the tree. A similar cessation of growth, followed by abscission, occurs in young apples whose seeds have been eaten by the larva of the sawfly (*Hoplacampa testudinea* Klug) or other insects. (Luckwill, 1954). In grapes there is a high correlation between seed weight and berry weight (Olmo, 1946). In the seeded banana, growth rate is strictly proportional to the seed content (Simmonds, 1953).

The final and most conclusive evidence came from the brilliant work on the growth of the strawberry fruit by Nitsch (21, earlier work quoted there). Auxin analysis showed the receptable without any of the hormone, while the achenes were rich in it. Growth of the receptable was decreased the more achenes ("seeds") were removed, and it stopped entirely when all the achenes were removed. This was not due to injury to the young fruit, for growth was resumed when synthetic auxins were supplied to the receptable after the operation, and completely normal sized fruits without achenes could be obtained artificially this way.

In flower buds of plants that produce parthenocarpic fruit naturally, Gustafson invariably found a higher auxin content than in their non-parthenocarpic counterparts (1939). This was recently substantiated by Nitsch et al. (1957). It could account for pathenocarpic fruit development.

Thus it would seem reasonable to conclude that normally seeds control fruit growth by releasing auxins and that auxins are the limiting factor in fruit growth. There are, however, a few disturbing observations which make one wonder whether things really are that simple.

Swarbrick (1948) several years ago found that estrogens cause parthenocarpic fruit set in tomatoes. Rood and Hamner (1954) noted that 4-phthalimido-2,6-dimethylpyrimidine is capable of setting tomatoes. This chemical is not an auxin either. Neither is N-1-naphthyl phthalamic acid which increased the yield of field-grown tomatoes

from 6 to 15 tons per acre when 25 ppm were sprayed to the whole plant (173a). Recently, Wittwer et al. (1957) and Rappaport (1956) set fruit with gibberellic acid. Low temperature often results in parthenocarpic fruit development. Do all these agents cause their effect on the ovary via auxin? Or are there alternate paths? In our analysis of the growth-promoting factors we have so far been looking only for auxins. Our techniques for measuring the auxins in plant materials are accurate, convenient and sensitive. I wonder what we will find when we have perfected techniques for measuring kinins, gibberellins, etc. in plant tissues.

BENEFITS OF RAISING LOW AUXIN LEVELS IN FRUIT ARTIFICIALLY

Auxins have considerable use in fruit culture. The basic principle is that whenever auxin becomes too low in the developing fruit, it can be brought up to the desired level by spraying with synthetic auxins. Such treatment must be made before damage resulting from low auxin levels has become apparent. It is a preventative treatment.

One of the earliest uses was to supplement pollination. Fruit-setting problems are common with greenhouse tomatoes because they are frequently grown when the days are short, the weather is cloudy, and light intensities are low. Under such conditions, little or no pollen is produced, and within the flower parts the pistil tends to elongate and extend beyond the stamen cones. The result is an initial deficiency of good pollen, coupled with a modified floral structure not favorable for self-pollination. The absence of wind and air movements under glass, which normally aid in pollinating field-grown tomatoes, further lessens the possibility of a good set of fruit (See Wittwer, 1954). Use of growth regulators as supplements to pollination has under modern cultural practices insured fruit set on greenhouse tomatoes. It has also increased fruit size. Water solutions of three synthetic auxins have been recommended, as these set fruit effectively without injury to the plant: β-naphthoxyacetic acid (50 ppm), *para*-chlorophenoxyacetic acid (XIII) (15 ppm), α-*ortho*-chlorophenoxypropionic acid (XII) (40 ppm). However, methyl and ethyl esters of indoleacetic acid, the natural hormone, can also be highly effective in stimulating parthenocarpy (Sell et al., 1953).

Outdoor tomatoes also can benefit from sprays with synthetic auxins to supplement pollination. Early in the season night temperatures

usually drop below 59°F. Although such a low temperature is favorable for flower formation, it is minimum for proper fruit setting. Pollen production, pollen germination, and growth of the pollen tube is poor under such low temperature conditions. Consequently, poor set results and those fruits that set are small because the relatively few seeds in them do not produce enough growth hormone. Consequently spraying the flowers with 30 ppm of *para*-chlorophenoxyacetic acid (30 ppm) improves both fruit set and fruit size. These benefits are apparent for tomatoes grown for the early market for table use. Synthetic auxins have not been used to a signficant extent, as far as I know, on the huge acreages of tomatoes grown for canneries. Usually temperature conditions are more suitable, earliness does not carry a premium as it does for table tomatoes, and total yield for the season is not increased with these auxin sprays. Recently, however, as we mentioned earlier, N-1-naphthyl phthalamic acid, a growth regulator which is not an auxin, has given large increases in total yield in field trials (173a).

If one knows the basic rules of auxin physiology, and has imagination and patience, one can come up with fascinating new uses for synthetic auxins. Thus, Crane (1954) had noticed that in the young apricots that drop on the ground after frost damage, the seeds were dead while the other fruit tissue was undamaged. Knowing that the developing seed is the center of growth hormone production, he reasoned that he could save the frost-damaged fruit by replacing the natural auxin source with an artificial one. Indeed, 40 ppm of the synthetic auxin 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (XXVII) sprayed on the trees as late as two days after frost damage occurred saved the crop. Ninety-eight percent of the frost-damage fruit grew to normal size after having been treated with synthetic auxin, while the untreated apricots were a 100% loss.

Even without frost damage, apricot fruits have a tendency to drop between the time of thinning and the time the fruit matures. The Stewart variety drops as much as 30%, the Tilton variety as much as 50%. This drop in the Stewart variety is also attributable to seed abortion. Crane succeeded in forestalling this fruit drop, too, with 2,4,5-T (25 ppm) sprays at the time pit hardening of the fruit begins. This is six weeks after full bloom, when the embryo averages one-half mm in length. Five years of experimentation with 2,4,5-T in California has

proven that the size of apricots can be increased as much as 45%, that preharvest drop can be eliminated, and that a red blush can be induced on the fruit under certain conditions (Crane, 1956; Bradley and Crane, 1957). Application of 2,4,5-T to hasten maturity, increase size and prevent preharvest drop is rapidly becoming a standard practice. The increased size of the fruit was entirely due to increased cell size; the number of cells had not increased as a result of the auxin sprays. The cell nuclei were also larger and more highly polyploid than those of the controls. The respiration rate declined and this persisted throughout the season in the fruit from treated trees (Maxie and Crane, 1956). This lower respiration rate and increased growth may mean a better energy coupling between respiration and growth. It is the opposite of the action of dinitrophenol which brings about uncoupling and which results in an increased respiration and a decreased growth.

A related type of auxin insufficiency which can be corrected by auxin sprays occurs in the apple. Luckwill (1953) has shown that when the seed matures it ceases to produce growth hormone. The resulting drop in auxin level in the fruit causes abscission, known in practice as preharvest drop of apples. Under particularly adverse conditions as much as 90% of the apple crop may drop to the ground. Some varieties are more susceptible to fruit drop than others.

In 1939 Gardner, Marth and Batjer showed that auxin sprays can prevent this loss. Already in 1942 "hormone spraying" of apples was becoming a standard orchard practice. Of this application, Auchter, then a Research Administrator with the U. S. Department of Agriculture, said at that time: "This single application should save far more than all the money spent on all our hormone research." Today, sprays with synthetic auxins are in general use for apples and pears, wherever preharvest drop is a problem (Batjer, 1954).

It is estimated that losses from fruit drop in apples and pears have been reduced 60 to 80%. In many instances an additional benefit is derived in the form of better fruit color and quality made possible by delaying harvest for a limited period of time. The reduction alone in the loss of fruit commonly knocked off in harvest operation would, in most cases, more than pay for the cost of the spray, which is about one cent per bushel (Batjer, 1954).

The preferred synthetic auxin is 2,4,5-trichlorophenoxyacetic acid.

Only 48 grams per acre, applied about a month before picking, keeps the apple drop down to 1% or less in the Pacific Northwest (Batjer et al., 1954). Recently, 2,4,5-trichloropropionic acid (2,4,5-TP) (XXXII) has also given excellent results. In New Zealand this material is being used in practice, and NA and 2,4,5-TP are most satisfactory materials there (Davison, 1956).

When preharvest drop in apples was first controlled, naphthaleneacetic acid was the chemical used most effectively; phenoxyacetic acids had not yet been tried. Since other crops, e.g., citrus, also suffered from preharvest drop, naphthaleneacetic acid was tried here also. However, this chemical was totally ineffective, and it was not until W. S. Stewart tried the new chlorinated phenoxyacetic acid, 2,4-D, that success was attained. Today spraying with minute quantities of 2,4-D has become a standard practice in the citrus industry. It has shown remarkable effects in increasing fruit size and in decreasing fruit losses from fruit and leaf drop. Storage life of lemons is increased by adding 100 to 1,000 ppm of 2,4-D to the wax emulsion applied during packing. This treatment prevents the "button" (stem plus calyx) from dropping off (physiologically, this is equivalent to controlling preharvest fruit drop) and thus preventing penetration of the *Alternaria* fungus (Stewart et al., 1952; Erickson, 1952; Hield and Stewart, 1956).

Another interesting and economically important use for synthetic auxins is in the holly industry. In fact, the holly growers in the Pacific Northwest owe their existence to synthetic auxins. The Pacific Northwest has an ideal climate for holly culture, but before the era of synthetic auxins its market was limited to a small radius. The product was so perishable that growers had to wait until a few days before Christmas and then rush it to the buyers before leaves and berries fell off. In 1937, Gardner and Marth discovered that synthetic auxins can prevent the leaf and berry drop of holly. For several years now, a naphthaleneacetic acid dip has been used which prevents abscission of both leaves and berries. This treatment allows growers to start shipping late in November, thus expanding the marketing radius, and now the Northwest produces most of the nation's holly (Syring, 1951). The principle upon which this application is based must be the same as that which stops preharvest drop in apples. The cutting

of holly branches reduces the auxin level. This is prevented by bolstering it with synthetic auxin.

If one plots growth of a fruit against time, one obtains a sigmoid curve in the simplest case. Growth of the ovary starts slowly in the flower, gains momentum after pollination, and slows down again when the seeds and fruit mature. Some fruits, apricot and fig for example, have a double sigmoid curve: two periods of rapid growth separated by a period of slow growth. This period of relative inactivity can be shortened by application of the synthetic auxin 2,4,5-trichlorophenoxyacetic acid (Crane and Blondeau, 1949) and fruit ripening is then speeded considerably. This discovery has found practical use in apricots, where maturation was speeded by as much as 18 days (Crane and Brooks, 1952). However, in figs neither hastening of maturation nor induction of parthenocarpic fruit in the Calimyrna variety is in commercial usage. The Calimyrna fig is normally pollinated by a wasp. The process (caprification) is cumbersome and can be bypassed by use of 4-chlorophenoxyacetic acid. Yet, the occurrence of "seeds" and their accompanying nutty flavor is so important to the trade that auxin-induced parthenocarpic Calimyrna figs have not proven a practical reality. Crane (1952) has used benzothiazole-2-oxyacetic acid (XXVIII) to induce parthenocarpy and has obtained drupelets with a sclerified endocarp (hollow seeds). However, there are still some difficulties to be overcome before the practice is adopted by the industry.

More successful, commercially, appears to be the use of synthetic auxins in replacing girdling in grapes. In certain grape varieties, such as Black Corynth (currant), girdling is required to set the berries. In many other grape varieties, girdling increases fruit size. *Para*-chlorophenoxyacetic acid (XIII) is now beginning to replace girdling in commercial grape production in many varieties in California (Weaver, 1956). Larger berries are produced with firmly attached pedicels, giving desirably compact fruit clusters. In South Australia more than 90% of the currant crop has resulted from spraying with *para*-chlorophenoxyacetic acid instead of girdling (B. G. Coombe, 1956, private communication). Similarly in Greece this auxin has found wide application in the seedless raisin industry (G. Karamanos, 1957, private communication).

It is found at times desirable to produce small sized Zante Currants,

such as for the baking industry. This has been achieved by applications of benzothiazole-2-oxyacetic acid (Weaver, 1956). Smaller berries might also be of value in regions where rot is prevalent. However, recently a much better way has been found to "open up" the fruit clusters by application of gibberellic acid. This growth regulator increases elongation of the stems in the clusters (Stewart et al., Weaver and Olmo, 1957).

Another interesting use of auxins is in blackberries. Each year several hundred acres are sprayed commercially in the San Joaquin Valley in California. Increases of 12 to 18% in fruit yield result from an auxin-induced drupelet size. The number of drupelets per fruit does not change. *Para*-chlorophenoxyacetic acid sprayed at full bloom at 50 to 100 ppm is the most effective auxin (Bringhurst et al., 1956).

AUXIN-INDUCED FRUIT DROP

There is another use of synthetic auxins, which is just the opposite of that considered above: promotion of the drop of young fruit. This "thinning" of young fruit is done for two principal reasons, to improve size and quality, and to produce a crop every year on trees that have the alternate bearing habit. Many kinds of fruit trees set a greater number of fruits than can attain marketable size. When this condition exists, it becomes necessary to remove the fruit surplus relatively early in the growing season. Hand thinning is becoming more and more impracticable as labor costs rise. A cheaper and more rapid method has been found in many instances in chemical thinning during or shortly after the bloom period. These thinning agents are not necessarily auxins; dinitro-*ortho*-cresol chemicals are being used extensively on both apples and peaches (Batjer, 1954). These chemicals are pollinicides and are used at full bloom. However, synthetic auxins also have found a use in thinning. Naphthaleneacetamide has been used with success in recent years for thinning apples, while earlier naphthaleneacetic acid was used (28). These materials are relatively stable in the plant and are not attacked by the IA oxidase system of the apple (Teubner and Murneek, 1955). In the United States one chemical or another is now almost universally used in apple culture, while in Britain fruit thinning by chemicals is not yet accepted as a commercial practice (Luckwill, 1957). One reason for this might be reports from England that, although thinning by NA is adequate and

the mean fruit weight is increased, no increase in total marketable crop is obtained (Abbott, 1954).

In addition to the successful use of thinning of apples by auxins in the United States, there is a similar successful commercial use in the olive culture in California (Hartmann, 1952). Alternate bearing is especially striking in this crop. One year there will be practically no fruits on the tree; the next year the tree is overburdened. On the year that the tree is overburdened the size of the olives is small and the only use for them is in olive oil. It is much more profitable for the farmer to sell his olives to the canneries, but the latter accept only large fruits. Naphthaleneacetic acid sprays applied a few weeks after bloom to the small fruits has provided the solution. Appropriate thinning is achieved. Fruit size is increased. The great differences in yield in alternate years is evened out.

The degree of thinning is proportional to the concentration used. High concentrations prevent fruiting altogether. This is used by gardeners in the Hollywood area on ornamental olives around swimming pools. Many other ornamental trees also produce undesirable litter when their fruits drop to the ground. This also can be prevented by NA sprays (Chadwick et al., 1951).

The physiological reasons for the auxin-induced fruit drop are not clear. It appears to be an exaggerated "June drop". Thus, Kelley (1955) reports that peaches treated with NA a month after full bloom dropped their fruit with the "June drop". The "June drop" on the treated trees began earlier and lasted longer than on the checks. Applications made too early to affect the "June drop" did not thin. Neither did applications during or after the "June drop" thin the fruit. Teubner and Murneek find in apples that one of the first effects of auxin thinning sprays is retardation of fruit development. The major portion of these retarded fruits eventually comes off during the "June drop" ("3rd drop").

What is the physiology of the "June drop"? The fascinating work of Luckwill has shown that the seed produces a regulator which is an anti-drop hormone. It is not an indole compound. It promotes growth of wheat sections, but much less than IA. It has a marked activity in the tomato parthenocarpy test. It is produced in the endosperm but not continuously. During rapid digestion of the endosperm its production falls and results in the "June drop". This anti-drop regulator

is called by Luckwill "Malus auxin 2", but the reviewer would like to submit the hypothesis that this anti-drop regulator is not an auxin but a gibberellin. If the properties of the anti-drop regulator as given by Luckwill are compared with those of gibberellic acid (for which the literature is quoted), the following similarities may be noted: both promote extension growth of wheat coleoptile sections, but much less than IA (Brian, Hemming and Radley, 1955); both excell in bringing about parthenocarpic fruit development in tomato (Wittwer, Rappaport); both are produced during certain stages of seed activity only (Ritzel, West and Phinney); both have an Rf slightly above that of IA, and the chromatogram of GA on leaf base sections looks very much like that of inhibitor 1 and auxin 2 of apple leaf extract on wheat coleoptile sections (van Overbeek, Racusen et al.); both are capable of preventing abscission of young fruit. Walhood, of the Cotton Research Station at Shafter, California, showed that very young cotton fruit could be prevented from dropping by application of gibberellic acid (Proc. 12th Ann. Cotton Conf., Memphis, Dec. 1957).

If we assume (*a*) with Luckwill that the "June drop" is brought about by a drop in the production of the anti-drop regulator and (*b*) that this regulator is a gibberellin, it becomes at once apparent why auxin-induced fruit thinning expresses itself as an exaggerated "June drop". It is at present becoming clear that gibberellin-controlled processes are strikingly antagonized by auxin. Hayashi and Murakami (1954) showed that the lower portion of the fifth growing leaf of oat responds to GA with growth increase. This GA-induced growth was counteracted by IA. By contrast, the upper part of the leaf responds to IA but not to GA. Here GA helps the action of IA. The leaf, therefore, goes through at least two growth phases—one GA-dominated, another IA-dominated. In our laboratory (van Overbeek, Racusen et al., 1957) we found that the youngest part of the shoot enclosed in the *Avena* coleoptile will only grow with GA and not with IA. IA strongly suppresses this GA-dominated growth. Walhood (see above) very recently provided the most conclusive evidence which makes it likely that auxin thinning in fruit is nothing but the auxin antagonism of a gibberellin response. He found that the natural drop of young cotton fruits can be stopped by application of GA and much promoted by application of IA. Cotton fruit abscission could be controlled at will by varying the relative concentration of these two regu-

lators. We agree entirely with Luckwill (1957, p. 79) that "It is clear ... that no longer can we think in terms of growth control by a single auxin such as IAA".

ROOT FORMATION

Soon after indoleacetic acid was discovered to promote cell elongation, it was found that it also promotes formation of root initials on cuttings (see Went and Thimann, 1937). This was the first function of auxin that was found outside of cell elongation. It was also the first time that a practical use was found for auxins. Synthetic auxins were found more useful for the induction of roots on cuttings than the native IA because the synthetic chemicals are more stable in the plant. Commonly used in horticultural practice are indolebutyric acid (III), naphthaleneacetic acid (IV) and a mixture of these two auxins. Recently, also, the chlorinated phenoxy acids, such as 2,4,5-TP (XXXII), have been found highly active as root initiators (Templeman, 1955). The widespread use of auxins by nurserymen, florists and other horticulturists indicates that they are valuable aids to rooting (Stoutemeyer, 1954). Treatment with auxins will not help root formation on cuttings that have no natural tendency to root. Use of auxins in rooting of cuttings therefore has real limitations. However, the following distinct benefits are the reasons for the economic success of auxins in horticultural practice: (*a*) There is usually a considerable saving of time, often amounting to well over one-third of the usual rooting period; since rooting is more rapid, there is less opportunity for the cuttings to deteriorate; (*b*) a larger percentage of cuttings form roots; (*c*) they have a compact root system composed of many short roots instead of the few long roots when the rooting "is left to nature".

One of the largest single uses is perhaps in the vegetative propagation of cacao with a mixture of indolebutyric and naphthaleneacetic acids. In all the West Indian Islands, as well as in West Africa, this has become a standard method in the propagation of cacao from cuttings. In rubber propagation the auxin method has found large commercial use (Templeman, 1955). In coffee, vegetative propagation by means of cuttings is practiced in India, Java, Africa and in Latin America (Fiester, 1957).

Successful root formation depends upon the presence of a number of co-factors in the cuttings which put the tissue in a reactive condition. Normally these co-factors are provided by leaves. It is well known

by people experienced in plant propagation that loss of leaves from cuttings in the propagating frame greatly reduces the chances for successful rooting. Thick cuttings, with much reserve material stored in them, do not need leaves. Analysis of the materials which the leaves provide has made it likely that sugars and nitro-genous materials are involved. Among the latter, arginine is highly active (see van Overbeek, 10, 24, 260; Leopold, 2).

WEED CONTROL

By far the largest use of synthetic auxins has been in weed control. The history of the discovery of this use is not entirely clear, since it was shrouded in the secrecy of wartime research. Audus (1953) gives the following account. Slade, Templeman and Sexton, working at the agricultural research station of Imperial Chemical Industries, Ltd., were investigating the probable part played by auxins in the beneficial effect of organic matter in soils. In the course of experiments in 1940 in which oats were grown in boxes in the open and sprayed with naphthaleneacetic acid solutions at the rate of 25 lbs./acre, the discovery was made that seedlings of wild mustard were killed by the auxin, whereas oats were unharmed. This indication that synthetic auxins could exert a selective toxic action on plants when applied at these high concentrations led to a series of planned experiments. It soon became apparent that cereals were relatively insensitive, wheras broad-leaved weeds were very sensitive to such treatment. The search for other chemicals that might be more effective produced 2-methyl, 4-chlorophenoxyacetic acid (MCPA) in 1941 and 2,4-dichlorophenoxyacetic acid in 1942 (Templeman, 1955). These experiments were not published until 1945.

According to Leopold (2), in the U. S., Kraus started investigating herbicidal qualities of auxins in the fall of 1941. In June, 1942, Zimmerman demonstrated on live material the auxin-like responses and growth abnormalties caused by 2,4-D at the Cold Spring Harbor Symposium on Hormones. However, at that time no mention was made of herbicidal properties, as far as I recall. It was not until late in 1944 that the successful use of 2,4-D as a herbicide was announced by researchers at the U. S. Department of Agriculture and The New York State Agricultural Experiment Station.

My own experience with 2,4-D began in the spring of 1945 when we started an extensive program to evaluate the use of 2,4-D as a

herbicide in the tropics. The results were highly successful from the start as can best be described by an account written in those early days of weed control (van Overbeek, 1947): In 1945 my associates and I had selected for weed control experiments a field in which sugar cane had recently been planted. The cane was about a foot above the ground, and an abundance of small weed seedlings gave the field a greenish hue. The weather was warm, as it usually is in Puerto Rico, and the soil was rich and contained ample moisture. When we returned two weeks later, a lush blanket of broadleaved weeds, some two feet in thickness, had all but covered the cane crop.

At that time, the field was sprayed with an aqueous solution containing 0.075% of the ammonium salt of 2,4-D. Two weeks later, when the plantation was revisited, the weeds were down and the cane was again free to take full advantage of its favorable environment. Half a dollar's worth of 2,4-D per acre had restored the balance in favor of the cane crop.

After a year of continuous field testing, possible only in the tropics, our experiences and recommendations were published in a comprehensive bulletin (van Overbeek and Velez, 1946). Sugar cane was obviously the tropical crop where 2,4-D would do the most good.

All over the world 2,4-D and MCPA were given trials and found highly effective, especially in the culture of cereals such as maize and the small grains. In 1948, just three years after the first field trials, an estimated 16,000,000 pounds of 2,4-D and related compounds were manufactured and sold at an average price of $0.75 per pound. According to a report by the U.S. Tariff Commission, 60,000,000 pounds were produced in 1953 (254). This represents some $30,000,000 at the manufacturer's level.

In the United States in 1952, 12% of the 139,000,000 acres of small grains were sprayed for weed control; 2,4-D was used almost exclusively. Of 82,000,000 acres of maize, 11% was treated for weed control. At present, combined annual weed control usage of 2,4-D, MCPA, 2,4,5-T and 2-(2,4,5-trichlorophenoxy)propionic acid is practiced on 30 million acres of cultivated land in the United States (Shaw and Gentner, 1957). In 1955 over 100,000,000 acres throughout the world received treatment with auxins as herbicides (32). 2,4-D and the related MCPA are used in Europe on many millions of acres of cereal crops (Woodford and Kasasian, 1956). In England,

MCPA is the preferred auxin herbicide; it can be applied early on oats without damage as is likely to result from 2,4-D (Elliott, 1956). Wain (1955) has further reduced toxicity and increased herbicidal selectivity by introducing the butyric acid homologs: MCPB and 2,4-DB (Table I). These are inactive as such but converted by some plants to the active MCPA and 2,4-D by beta-oxidation (see below).

What benefits are derived from the use of herbicides? Willard (1954) mentions that in 1930, as an average of all wheat marketed at Minneapolis, between 7 and 8% was weed seed. Such contaminated grain is now a thing of the past because of the universal use of herbicides. Figures obtained in England and Europe show some 20% gain in yield (Woodford and Kasasian, 1956). In Canada in 1949, 8,000,000 acres of grain sprayed with 2,4-D produced an estimated increase of from three to five bushels per acre. This gain amounts to an additional yield of from 35 to 40,000,000 bushels of grain on the same acreage simply by the application of herbicidal chemicals (24,208). In India a 34% increase in wheat yield from the use of MCPA has been reported (Row, 211). The same chemical gave an increase of 75% in rice yield in California.

What were the reasons that the use of 2,4-D was so successful? The first reason was that 2,4-D was truly selective; it did something which was not possible before. The alternative of chemical weed control is mechanical weed control, and this is impossible in grain fields. It so happened that cereal plants were much less sensitive to auxin herbicides than broad-leaved weeds, which were the major pests.

The second reason was that this unique new type of weed control was highly effective at a low cost. In 1952, 16,000,000 acres of small grain were sprayed for an average cost of only $0.66 per acre for chemical (60).

Third, of course, this weed control was achieved in a major food crop, the cereals, which created a large market for the chemical.

Use of 2,4-D as a herbicide is not restricted to grain crops. We mentioned sugar cane already, a major crop in the tropics. Brush control with 2,4-D, 2,4,5-T and 2-(2,4,5-trichlorophenoxy)propionic acid (XXXIII) is used on a large scale. Brush control is practiced on railroad and utility rights of way, along highways, and also in agriculture to improve rangeland by replacing brush with grass,

thereby improving the animal-carrying capacity of the land. Studies on the control of sand sagebrush *(Artemisia filifolia)* conducted by the U. S. Southern Great Plains Field Station have shown that the cost of one single treatment with 2,4-D at $2.50 per acre is returned every year for ten years. In a similar study of six years duration, beef production was increased 70% (31).

In the decade since its introduction into agriculture, much experience has been gained in the handling of 2,4-D. A material having the high physiological activity it has must be handled with caution to avoid damage to sensitive crops. Fortunately, 2,4-D is nontoxic to man and animals. Man's use of 2,4-D can be likened to man's use of fire. Special precautions are necessary in both cases for beneficial use. If these are ignored, damage results.

In the early days of 2,4-D there were frequent reports of crop damage due to contaminated spray materials. Drums in which 2,4-D was originally shipped were used for other agricultural chemicals. Spray rigs were not properly cleaned. Today, one does not hear about these troubles anymore, for we have learned how to handle 2,4-D safely.

Another cause of 2,4-D damage was wind-borne contaminants. For instance, when grain fields were sprayed with 2,4-D, the chemical would collect on dust particles which would then be blown by winds and contaminate cotton fields or vineyards miles away. This has led to all sorts of regulations concerning the use of 2,4-D. Like many other laws, some are wise, some seem exaggerated and foolish. In the latter category, those laws made during the early days of 2,4-D would almost certainly need revision. As with fire, it seems wiser to teach people how to use it than to forbid its use altogether.

Not only was experience gained in the safe handling of 2,4-D but also in its application to new crops and weeds. While originally, 2,4-D was sprayed in low concentrations on the foliage of gramineous crops and dicotyledonous weeds, it was found in sugar cane, for instance, that higher concentrations could be sprayed on the soil before the crop was above the ground and that in that way grassy weeds also could be controlled (For tropical weed control, see van Overbeek, 1954).

When airplane spraying gained in popularity, it became essential to increase the concentration of 2,4-D solutions; it was not long before formulations were found in which esters of 2,4-D in oil car-

riers were sprayed at a rate as low as one gallon per acre. Even when only a drop of solution falls on a weed, it will be killed, as the 2,4-D is translocated throughout the plant. This, however, is potentially dangerous to crops growing in adjacent fields, where damage contributed to restrictive legislation. It is generally thought that the esters are hydrolyzed inside the plant and that the 2,4-D is the physiologically active form.

Since this is a discussion of auxins, the auxin herbicides have been stressed. But it should not be forgotten that only a fraction of weed control is achieved by auxin herbicides. This fraction is an important one, but many more weeds are being killed each year by cultivation than by chemical means. In fact, a farmer prefers to control his weeds by mechanical means if this is at all possible because mechanical weed control is usually cheaper than chemical weed control. It is in cases where mechanical control is impossible, as in grain fields, or expensive, where mechanical cultivation must be supplemented by hand hoeing, that chemical weed control attains its true significance.

Furthermore, although the auxin herbicides are at present the most important among herbicides, there are many other chemicals which are used with success for weed control purposes. Among these the dinitro chemicals rank high. They were discovered before the auxin herbicides and are still used rather widely in cereal crops. In The Netherlands, for instance, where both winter and spring cereals are grown, 3,5-dinitro-o-cresol is used almost exclusively and the percentage of the cereal acreage sprayed with this chemical varies from 80-90 for oats and winter rye down to 20-40 for winter barley and wheat (Woodford and Kasasian, 1956).

Then millions of gallons of herbicidal oils are used to control grasses and other herbaceous weeds along roads, fences and canals. Some of the lighter oils are selective and are used to remove grass from beds of celery, carrots or pine seedlings. The mode of action of these materials is based upon solubilization into the plasma membrane (van Overbeek and Blondeau, 1954).

Millions of pounds of inorganic herbicides are used to prevent weed growth in storage yards of factories, etc. These materials are chlorates mixed with borates to reduce fire hazards. The chlorate-

borates are also used to remove foliage from cotton prior to picking by machine.

Recently, several new types of herbicides have been introduced. These are of considerable interest, but they have not as yet found the widespread use that the auxin herbicides have. Among these new materials may be mentioned ureas and chlorinated propionic acid. Among the former are 3-phenyl-1, 1-dimethylurea (fenuron) and chlorinated derivatives 3-(p-chlorophenyl)-1, 1-dimethylurea (monuron), 3-(3,4-dichlorophenyl)-1, 1-dimethylurea (diuron), and 3-(3,4-dichlorophenyl)-1-butyl-1-methylurea (neburon). The toxicity to plants of some of these ureas is as high as that of the auxin herbicides. However, they generally do not appear to possess the high degree of selective toxicity that the auxin herbicides have. Most interesting among the new herbicides is 2,2-dichloropropionic acid (Dalapon). It is specifically toxic to grasses and is thus the counterpart of 2,4-D and MCPA which display little toxicity to grasses.

PHYSIOLOGY AND BIOCHEMISTRY OF 2,4-D

At the conclusion of this chapter we will now discuss some aspects of the physiology and biochemistry of 2,4-D. This will increase our knowledge of the action of this material, but it will fall short of giving us an answer on the precise mechanism of the toxic action of 2,4-D and on the basis of its selective toxicity. We could almost say the same of this field as Szent-Györgyi has said of muscle physiology. The more we know about it, the less we understand, and it looks as though we would soon know everything and understand nothing.

Before 2,4-D or, for that matter, any chemical can act in a plant, it has to penetrate into it. For this reason many research workers have given much thought to the problem of penetration. A summary of our knowledge has recently been published (van Overbeek, 25). Weintraub et al. (1954, 1956) have used radioactive 2,4-D and studied the factors that affect entry into leaves. Surfactants help penetration a great deal. This is undoubtedly largely due to increased spreading and wetting which bring about increased sorption of a chemical.

On studies with isolated cuticle it turned out that there was relatively little penetration of 2,4-D through the cuticle from dorsal, or stomatefree, surface of the leaf. This result was observed consistently in several experiments with cuticles of *Hedera helix* and *Clivia nob-*

ilis, with water or alcohol as solvents, and regardless of the pH or the presence of surfactants. However, that should not be interpreted to mean that in intact leaves there is no penetration in any species through the dorsal leaf surface. It does show that stomata are an important factor and that the lower leaf surface is the preferred site through which chemicals enter. Age of the leaf is also important. The youngest leaves absorb 2,4-D best, the oldest least. Rate of absorption of 2,4-D is not dependent upon light, but transport out of the leaf to the rest of the plant is. Turgid plants absorb 2,4-D more rapidly than dry plants. No difference was found between entry of radioactive 2,4-D into a grassy leaf (millet) and a bean leaf. Also, metabolizing of 2,4-D is not different in susceptible and non-susceptible plants. However, migration of 2,4-D out of treated leaves show excellent correlation with susceptibility. For instance, 25% of the applied dose migrated out of bean, soy bean and cotton leaves in 24 hours, but only 1% out of oats, wheat and rice leaves (Weintraub et al., 1956). This was confirmed by Ashton (1958); a surgarcane leaf retained 7x as much of the applied radioactive 2,4-D as a bean leaf. The growing point of sugar cane consequently received only one-tenth as much 2,4-D as the growing point of the bean. Recently, Crafts published his extensive investigations on translocation of 2,4-D (1956). The chemical moves out of the leaf via the phloem and is tranported in this tissue together with food materials. Of course, when 2,4-D is presented to the roots, it moves upward with the transpiration stream via the xylem. Hay and Thimann (1956) found that 2,4-D translocation in bean ceases after 24 hours, due to the toxicity of the material. They doubt that intact 2,4-D gets to the roots. However, Ashton (1958), using a more sensitive technique, did demonstrate that intact 2,4-D moved down from the foliage into roots of sugar cane and of beans.

Weintraub et al. (1952) determined the radioactive carbon dioxide which was produced by bean plants given radioactive 2,4-D labeled with C^{14} at the C_1 position of the side chain, the C_2 position of the side chain, and the C_1 position of the ring. The CO_2 which shows up fastest comes from the C_1 of the side chain. Within 24 hours about 1.7% of the original C^{14} in 2,4-D was back in CO_2. This came from the first carbon atom. Later, increasing amounts of CO_2 also came from the second carbon atom in the side chain. No carbon from the ring turned up as CO_2. The plant, therefore, starts to break down 2,4-D

by breaking down its side chain. About 1% per day is metabolized in this fashion.

Wain and his collaborators (Fawcett et al., 1956) reported that when 2,4-D is supplied to flax seedlings through their roots, and the plants steam-distilled after a suitable interval, phenol in appreciable quantities turns up in the steam distillate. It was found that also higher homologs with an even number of side chain carbons gave rise to phenols. On the other hand, acids with uneven numbers of carbon in the side chain yielded only traces of phenol. These results confirm earlier observations by Synerholm and Zimmerman (1947) that the side chain is degraded by beta-oxidation. Stumpf and Barber have recently (1956) reported that the site of beta-oxidation of a large number of saturated fatty acids is in the mitochondria.

Fawcett et al. exposed solutions of a series of 2,4-dichlorophenoxy alkanecarboxylic acids to the enzymatic actions of wheat coleoptile or pea epicotyle tissues. Ether extracts of the solutions were then chromatographed and analyzed by bioassay. Whether pre-treated with wheat or with pea tissues, assessment of the chromatogram segments revealed the formation of 2,4-D from the butyric and caproic acids (even numbers of C in the side chain) but not from the propionic, valeric and heptanoic acids (uneven numbers in the side chain). These results also are readily explainable on the basis of beta-oxidation. When the corresponding amides were tested in the same fashion, similar results were obtained. Evidently the amide was first converted to the acid by the tissues and the acid produced then underwent beta-oxidation.

When the nitriles of these acids were tested, a different behavior was encountered. Pea stem tissue was capable of hydrolyzing only 2,4-dichlorophenoxyacetonitrile, not the higher members of the series. Wheat tissue, on the other hand, was capable of hydrolyzing all the nitriles, and in all cases 2,4-D showed up. Nitriles can thus be degraded with the loss of one carbon atom. It is, therefore, clear that alpha-oxidation of nitriles and beta-oxidation of acids occur in plants. These reactions are also known in animal biochemistry.

2,4-D and related acids are also extensively broken down in the soil by micro-organisms. This is of practical importance, as it prevents building up of toxic levels of 2,4-D in agricultural soils. Adaptive

enzymes appear to be involved in the decomposition of 2,4-D and MCPA by soils (Steenson and Walker, 229, 276).

In addition to being broken down inside the plant, 2,4-D also appears to form a neutral complex with some cell constituent (Audus and Thresh, 1956). The plant evidently possesses a number of mechanisms by which it can inactivate 2,4-D. It is significant to remember, however, that inside the plant 2,4-D is vastly more stable than free indoleacetic acid. This was recently again clearly demonstrated by Andreae and Good (29a).

After 2,4-D has entered the plant, how does it affect the plant's normal metabolism? Since 2,4-D is an auxin, one might first ask, how does it affect the native auxin, indoleacetic acid? Audus and Thresh have attempted to give an answer to this question by treating sunflower and broad bean shoots with herbicidal levels of 2,4-D. After a reaction period of 24 hours the plants were extracted and the native indoleacetic acid analyzed chromatographically. There was no change in the native IA level as a consequence of 2,4-D treatment. However, the total level of physiologically active auxin must rise, as 2,4-D is an auxin. In the normal plant such a rise in auxin level is compensated by activation of the indoleacetic acid oxidase system, an inductive system. However, since 2,4-D is not attacked by this system and has been proven to be far more stable in the plant than IA, the auxin level stays high. This must mean that normal auxin metabolism is replaced by an abnormal one, caused by an excessively high auxin level. This in turn brings about changes in enzyme activities. We know of many such auxin-induced changes in enzyme systems; they are all indirect, as auxin has no effect on the isolated system. These changed enzyme systems, plus the resultant changed ratios between metabolites, are the intermediate steps between 2,4-D-induced abnormal auxin metabolism and the readily observable symptoms of 2,4-D toxicity. Since, if one accepts this view, the high level of auxin in the plant is the cause of herbicidal effect, it follows that any stable auxin of high activity is potentially a herbicide. This agrees with observed facts. One will recall that the herbicidal effect was discovered with indolebutyric and with naphthaleneacetic acid.

It may be interesting to mention at this point a number of observations which reflect the changed metabolism in a plant under the influence of high levels of auxins, specifically 2,4-D. Swanson et al

(1946) reported recently that triple crystallized 2,4-D gave rise to the formation of a red pigment, not an anthocyanin, in slices of tubers of *Helianthus tuberosus*. Other auxins, including IA, caused pigment formation too. Payne, Fults et al. (1953) have found that when potatoes are sprayed with 2,4-D in the field at the time the tubers are rapidly enlarging, the tubers show increased anthocyanin content at harvest time. This is desirable for marketing the McClure variety. Spraying 2,4-D also increases protein content of potatoes (Yasuda et al., 1955). Davis and Dimon (1953) found that auxins, e.g., IA and NA, reduce Fusarium wilt of tomato. This was attributed to a changed metabolism of the host plant. When *Vicia faba* was dipped in 5 ppm. 2,4-D, its susceptibility to *Botrytis fabae* infestation was reduced. It was suggested that the 2,4-D-induced sugar depletion in the host plant might account for its disease resistance (Mostafa, 1956) Treatment of tomato plants with *alpha*-methoxyphenylacetic acid induced in the root the appearance of some agent that had a repellent effect on nematode larvae (Wieser, 1956).

Japanese beetles prefer auxin-treated foliage. Sugar-cane borers prefer canes treated with 2,4-D. This is an especially interesting observation, since 2,4-D has no visible influence on sugar cane and the plant is resistant to the action of 2,4-D. It does show that 2,4-D penetrates a resistant plant, and that, therefore, restricted penetration of 2,4-D is not the reason for resistance to this chemical. Field mice find corn treated by pre-emergence applications of 2,4-D more palatable. In California *Centaurea solstitialis* became palatable to sheep after treatment with 2,4-D, and poisonous *Conium maculatum,* usually avoided by cattle, became palatable when treated with 2,4-D, with the result that the cattle died (see summary in van Overbeek, 1954). There can be little doubt that auxins change the pattern of a plant's biochemistry.

Recently attention has been called to the possible significance of inhibition of the transport of IA by 2,4-D as part of the herbicidal action of 2,4-D (Hay, 1956; Zwar and Rijven, 1956). However, 2,4-D is by no means the only chemical that inhibits IA transport. Sulfhydryl poisons and dinitrophenol inhibit IA transport at concentrations which do not inhibit respiration (Niedergang and Leopold, 1957). Auxins such as 2,4-dichlorophenoxypropionic acid and 2,4-DB, and anti-auxins such as 2,4,6-trichlorophenoxyacetic acid all inhibit IA transport by competing with IA at the carrier sites (306). Conversely the "for-

mative" response to 2,4-D can be completely prevented by application of IA (Weintraub, 1950).

In summary, one might conclude that the herbicidal action of 2,4-D and related materials is due to its high auxin activity, its relatively high stability in the plant and its high tenacity in the surface of the cytoskeleton. The selectivity of 2,4-D may be in part due to its lack of translocation out of leaves of resistant plants.

CONCLUDING REMARKS

Auxin physiology as such is three decades old, starting with the isolation of the growth hormone from the plant by Went in 1926. Went himself wrote the first article on auxins in The Botanical Review (1935) and a supplement a decade later. Reviewing the achievements in this field during the third decade, the following stand out. The most dramatic change from the previous two decades has been the use of auxins in agriculture. This development, especially the use of 2,4-D as a selective herbicide, took place almost entirely in the third decade of auxinology. This use of 2,4-D and related material has proven itself and is likely to stay. Its importance to agriculture is equal to that of the development of insecticides and of Bordeaux spray. The development of the auxin herbicides was not only significant as such, but has greatly stimulated industrial and agricultural research in agricultural chemicals.

In the pure physiological aspects of auxinology, the third decade has seen the adaptation of chromatography to the study of plant hormones. This has led to the characterization of indoleacetic acid and its immediate derivatives as the major naturally occurring auxins.

The fourth decade will undoubtedly witness the development of two new classes of growth regulators. the kinins and the gibberellins. Both of these materials are probably hormones of higher plants, and as such there must be interplay with the auxins. The interaction of hormones probably determines the physiological response of a tissue. It is certain that exciting discoveries will be made in this field of plant physiology. The golden age of plant hormones has just begun. Only by intensive research in fundamental physiology, including surface chemistry and biochemistry, by patient and generous financial support of such research, and perhaps by a little serendipity, can this golden age be fully realized.

ADDENDA

HORMONE INTERACTIONS

Recently J. Kato (Physiol. Plant. **11**:10. 1958) studied the interaction of gibberellin with auxins. Using gibberellin A and naphthaleneacetic acid, he demonstrated that the gibberellin promotes growth of the lateral bud. An equal dose of the auxin, simultaneously applied, completely prevented this gibberellin-induced growth of the bud. When the dose of gibberellin was increased five-fold, gibberellin promoted bud growth again, inspite of the presence of the auxin. Clearly, there is a balance between bud growth promotion by gibberellin and bud inhibition by auxin. Kinins also may be involved, as the recent studies of Wickson and Thimann (Physiol. Plant, **11**:62. 1958) indicate.

PRIMARY AUXIN REACTION

Three papers have become available recently which have brought to light some surprising facts that should help much in the understanding of the primary auxin reaction. It has been shown (306a, 306b) that as far as ring substitutions are concerned, phenylacetic acid resembles benzoic acids more than phenoxyacetic acids. Thus the 2,3,6-trichlorophenyl acetic and benzoic acids are highly active, while 2,3,6-trichlorophenoxyacetic acid is inactive. In order to attempt to understand why this is so, we postulate that the primary function of the auxin molecule is to provide a missing link in a H-bond network. It is the function of the acid group of the auxin to serve as such a link. It acts, as it were, as a "knife switch", closing a circuit, making the H-bond network oscillate (134). In order for the acid group to act as such a switch, it must be located just right and, furthermore, must be "insulated" electronically from the ring system of the auxin molecule. The ring system is merely a bulk with the function of anchoring the acid group exactly in the right position. The system is visualized in Fig. 2. It will be seen that the carboxyl group of the phenoxyacetic acid (left) is located too high above the H-bond network. This is a result of the di-ortho substituents of this molecule. On the other hand, this same substitution locates the acid group in both the phenylacetic acid (center) and benzoic acid (right) just in the right position. The orientation of the side chain out of the plane of the ring is another aspect of the correct positioning of the acid group. Obviously the H-bond network cannot be directly above

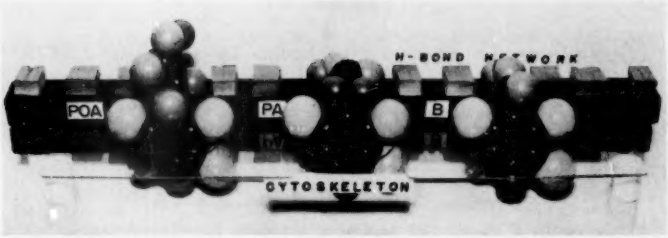


Figure 2. Comparison between models of molecules of 2,3,6-trichloro substituted phenoxyacetic (POA), phenylacetic (PA) and benzoic (B) acids as they are envisioned to fit into the cytoskeleton and its accompanying layer of H-bonds. Because of the two bulky chlorine atoms on both *ortho* positions, the rings have penetrated only relatively shallowly. The chlorine in the 3-position (below the plastic) helps in anchoring the ring. This shallow penetration locates the carboxyl groups of PA and B just right for becoming part of an oscillating H-bond system at the surface of the cytoskeleton, indicated by the square pieces of foam rubber. PA and B are therefore active. The sidechain of POA is too long for such shallow penetration in the cytoskeleton; it protrudes too far and its carboxyl group is out of reach of the H-bond network. POA is therefore inactive.

the cavity into which the ring fits; it must be to the side of it. This explains the third requirement for auxin activity that the side chain must be outside the plane of the ring.

In the other recent paper (306c) it was shown that the activity of naphthoic acids is increased dramatically by saturating the bond next to the carboxyl group. This of course forces the carboxyl group out of the plane of the ring, thereby *a*) positioning it for participation in the H-bond network and *b*) removing it from the resonance of the ring system, i.e., "insulating" the carboxyl group, making it less ionized and more capable of participating in H-bonding. It now becomes clear why 2,3,6-trichlorobenzoic acid is an active auxin: A) The two bulky *ortho* substituents force the carboxyl group out of the plane of the ring; this has the following consequences: *a*) it brings the carboxyl group within "reach" of the H-bond network; *b*) it "insulates" the carboxyl group from electron movements from the ring. B) The two bulky *ortho* groups prevent the molecule from sinking into the cytoskeleton too deep. C) The chlorine in the 3-position helps in anchoring the ring by van der Waals forces to the cytoskeleton and helps to reduce the likelihood of the molecule being thrown out of its active position in the cytoskeleton.

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MITOSIS AND METABOLIC ORGANIZATION[1]

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INTRODUCTION

This review is addressed to the problem of interpreting the nature of metabolic events associated with mitosis. Such events may initiate, support, or be a consequence of the process; but they all share the property of reflecting a physical reorganization of the cell more extreme and more extensive than most events in its history. It is, then, the relationship between structural and metabolic changes that constitutes the substance of this communication.

Cytology and metabolism have had a long history of separation; their association is less than 30 years old. Fleming's resolution of mitosis as a longitudinal splitting and separation of chromosomes—a milestone in the history of cell biology—was published in 1882 (6). Ten years later Eduard Buchner marked the start of modern metabolic studies by demonstrating fermentation in yeast extracts. But for nearly 40 years after Buchner made his fruitful discovery, physical chemistry and not catalysis was at the heart of cellular research. In 1922, Wilson, cutting the broadest of swaths in his survey of the cell, concluded that "life as we know it is indeed inseparably bound up with matter in the colloidal state. This conception seems likely to prove as fruitful in cytology as it has been in physiology" (114). In no less positive a way did the historian of biology, Nordenskiold, sum up the scene at that time: "That branch of chemical research which has had its greatest success in our own day and has excited the keenest interest is undoubtedly physical chemistry; each stage of its progress has at once been applicable to the living substance" (73). Such was the dominant view of cell biologists ten years after Warburg (105) had demonstrated that the oxidative capacity of liver cells could be separated in the form of sedimentable particles.

To trace the beginnings of cytochemistry, young though it is, one must leap well across the many years during which cell physiology found its most forward expression in terms of colloidal chemistry. In 1871, ten years before mitosis had been resolved, Friedrich Miescher

[1] Contribution No. 388, Chemistry Division, Science Service, Ottawa.

prepared nucleoprotein from nuclei of pus cells by digesting away the cytoplasm with pepsin and hydrochloric acid (66). The line of nucleoprotein studies thus begun was perpetuated by biochemists as outstanding as Hoppe-Seyler, Altmann and Kossel. For many years cell physiologists sought to apply the knowledge of nucleoprotein chemistry to vital and post-mortem staining, presumably because the acidity of the nucleic acids and the basicity of their associated proteins lent themselves readily to colloidal explanations. But cellular studies of nucleoproteins were also dotted with attempts to discover patterns of dynamic behaviour. By 1910 Oes had used a nuclease for digesting chromosomes, and around that time biologists were already regarding the differences in staining properties of dividing and non-dividing cells as indicators of changes in nucleic acid or protein (114). Many staining procedures were devised during that period, of which the most important to modern cytochemistry has been the Feulgen nuclear reaction; as a result of Feulgen's studies it became possible to establish the localization of deoxyribonucleic acid (DNA) in the chromosomes. Yet not until the end of the thirties did Brachet modify classical staining techniques (11) and Casperson perfect ultra-violet photomicrography so as to provide the first evidence of a dynamic relationship between nucleic acid function and the synthesis of proteins (16). Thus, 70 years after Miescher had isolated nuclei and nucleoproteins, cell physiologists turned their attention to linking cytology with established biochemical conceptions.

In this same period many of the rich disciplines of metabolism were introduced into cell physiological studies. Linderstrom-Lang and Holter developed extremely sensitive manometric and microchemical methods which made possible respiratory and chemical measurements of single cells (56). Others stratified animal eggs into clear and granular regions by centrifugation in order to determine the distribution of metabolic activities (11). A most important contribution was made by Bensley and Hoerr, who developed the technique of differentially centrifuging disrupted cells (86). This method, coupled with the application of electron microscopy to the identification of cell parts, has led to the extensive information we now have on the localization of cellular metabolic systems.

It would be wrong, however, to consider the above advances in technique as alone providing our present approach to cell physiology.

Somehow, in the course of the past 20 years a vigorous wave of interest has been set in motion, to which nearly every appropriate scientific discipline is responding. The impact of these approaches on our conceptions of cell metabolic organization is yet to be measured. Commonly, there is an implicit acceptance of the views already held by Remak and Virchow who before the turn of the century had put the final corrective touches on the cell theory of Schleiden and Schwann (6). How justified this acceptance is remains to be learnt. It is important, nevertheless, to examine our views on the nature of cell organization, for they have a considerable bearing on our interpretation of the metabolic patterns associated with mitosis.

CELL ORGANIZATION

Thirty-five years ago our present conception of cell organization was ably summed up by Wilson thus: "The difference between the cell and even the most intricate artificial machine still remains too vast by far to be bridged by present knowledge . . . nevertheless we accept the difference as one of degree rather than of kind, because it has proved itself fruitful of discovery and has kept us moving in the right direction" (114). Much of contemporary cytochemical research follows the assumption that the key to cell organization lies in an adequate cataloguing and summation of the contribution each cellular subunit makes towards the metabolic operation of the cell as a whole; and beyond that, in defining each cellular activity in molecular terms. Neither Whitehead's philosophical thinking on the prime importance of pattern (112) nor the assorted speculations of some biologists on the inadequacy of classical mechanics in characterizing living processes (113) have visibly influenced the arithmetic approach to cell behaviour. Rightly or wrongly, we have shown comparatively little interest in theory of organic units and a tremendous avidity for resolving cellular reactions and subcellular structures in molecular terms.

It is common to regard the past two decades of cellular research with extreme satisfaction. Our references to cellular components continue to acquire increasing precision in terms of metabolic activities and chemical composition. The basophilic elements of the cytoplasm —the endoplasmic reticulum of the electron microscopist or the microsomes of the cytochemist—are recognized as the ribonucleoprotein

structures intimately associated with protein synthesis. The mitochondria of the classical cytologist are more fully described as lipoprotein bodies housing the principal respiratory enzymes of the cell. With respect to the nucleus, a major advance has been the identification of deoxyribonucleic acid with genetic continuity. We may have also acquired some sophistication of language by trading the term "gene carrier" for "information center." Less progress has been made in elucidating the chemistry of the remaining subcellular elements; this gap of ignorance will probably be narrowed long before current cytochemical approaches will have run their course.

In light of the success achieved in viewing the cell as an integrated collection of metabolically specialized units, it might seem appropriate to discuss the problems of mitotic metabolism in terms of a mobilization of subcellular metabolic units to meet the manifold requirements of mitosis. In recent years, however, some features of cell organization have come to light that do not blend easily with this picture. Although these features have not compelled any novel conceptions they have served to shift our emphasis on what factors are significant in determining behaviour.

The principal shift of emphasis has been from the cell as a morphological unit to the cell as a genetic system. Mutation and self-duplication have become commonplace terms, even in biochemistry. There has been an enthusiastic revival of an old but unproven picture of the cell as an association of autonomously duplicating units, each with a genetic continuity of its own. The hesitation of some biologists in the nineteen hundreds to accept the idea that the nucleus is the exclusive seat of cell heredity, has been given substance in the accumulating evidence for extra-nuclear inheritance. And although the full issue is by no means resolved, it is now reasonable and acceptable to view the cell as an hierarchical association of genetical systems. According to this view, the metabolic specialization of a subcellular unit (the chloroplast, for example) is but one facet of its role in cellular organization. A second facet is the pattern of its perpetuation which has far-reaching physiological consequences. Thus, the chromosome, although no longer regarded as the exclusive seat of genetic transmission, stands out because of its extreme linear differentiation and its related economy of number. This high degree of organization is associated with a mechanism for equivalent division of progeny in

successive generations which has substituted the certainty in a rigid mechanical distribution for the probability in random segregation of large numbers. The reduction of gene types to as little as one or two per cell, coupled with the preciseness of mitotic segregation, accounts for the success of genetic analyses and is the reason for the predominance of the chromosome theory of heredity. But, underlying the pattern of differential inheritance for which the chromosomes are largely responsible, there are the lines of genetic continuity, less elaborately organized, which have their seat in the cytoplasm.

Which of the cytoplasmic constituents are autonomously transmitted remains unknown. It is certain, though, that the chloroplasts of many unicellular and higher plant forms, some oxidative elements of yeast, the kinetosomes of protozoa and the kappa particles of *Paramecium,* are among the cytoplasmic elements that cannot arise *de novo* (23). Some essential parts of their structure or some essential structure-forming parts must therefore perpetuate themselves in the cytoplasm. There may also be non-particulate cytoplasmic elements which are transmitted autonomously; the plasmon of *Epilobium* is a widely studied instance of this kind (65); so are the factors determining bilateral symmetry in desmids (108, 109).

The facts of cytoplasmic heredity are not discoveries of the past decade. Already in 1881 Schimper (114) concluded that plastids have an autonomous line of descent, and in 1908 the embroyologist Meves presented strong arguments for lines of inheritance via the cytoplasm (63). It is, however, doubtful that the comparatively slim knowledge of cytoplasmic heridity, even when very much strengthened by the lines of evidence recently brought together by Ephrussi (23), would themselves have been sufficient to influence the prevailing view that the cell has assigned its hereditary functions to the nucleus. A major and effective contribution to our thinking on cell organization has been provided by biochemical studies of viral infection and certain aspects of microbial genetics.

The chain of research beginning with the crystallization of tobacco mosaic virus and culminating in the demonstration that ribonucleic acid is by itself capable of maintaining genetic continuity has established the point that "self-duplication" is not an exclusive attribute of DNA (90). Its localization in the nucleus can therefore no longer be considered as evidence for an exclusively nuclear heredity. On the

other hand, the evidence for genetic autonomy of viral RNA has by analogy provided a basis for considering cytoplasmic RNA units as carriers of heredity. This has led to crystallographic and chemical studies of RNA in order to determine whether its structure is similar to the double helical one postulated for DNA (20). It has also stimulated studies to determine whether inter-specific transfers of genetically active RNA can be effected (50).

There are a few instances of cell organization in which cytoplasmic organelles are identical with infective agents, and these too have lent support to the idea that inheritance is not a purely nuclear function. In France, a number of years ago, L'Heritier discovered two geographical races of *Drosophila* which owed their peculiar distribution to differences in sensitivity to atmosphereic CO_2. His failure to obtain a Mendelian pattern of inheritance upon crossing the two races led him to discover that CO_2 sensitivity was due to a non-pathogenic virus. Since this virus was transmitted through the egg and, in the case of some mating types, even through the sperm, CO_2-sensitivity was perpetually reproduced as a racial characteristic. With this knowledge it was possible to create a sensitive race by injection of isolated virus, and thus to provide a striking demonstration of how the distribution between virus and cytoplasmic particle can lose meaning (55). On the basis of these studies L'Heritier concluded that viruses could be integrated into cell function without loss of autonomy. To this may be added the obvious inference that integrity of cell function is compatible with an autonomy of subcellular units. What is indigenous or foreign to a cell depends upon degree of integration: at one extreme are the pathogenic viruses, at the other the obligatory components of the cytoplasm. Between these extremes lie subunits which would appear to fall into the class of viruses, if capable of survival through an extra-cellular phase, or into the class of facultative cytoplasmic units if their loss from the cell is irreversible but not lethal.

The demonstrations that genetical determinants of the DNA type could be transmitted independently of organized chromosome structure have had considerable influence on our conception of cellular architecture. The transfer of heritable characters in *Pneumococcus* by means of chemically purified DNA is too well known a story to need retelling. Equally significant, though less well known, is the discovery of trans-

duction (54). In the latter phenomenon DNA is transferred from one bacterium to another by means of a virus which attaches to itself some hereditary determinants of one host and gives them up when infecting another. In both types of transfer there is a separation of genetical substance from its organized reproductive unit without impairment of its transmissibility. Whether reproducibility is also unimpaired, we do not yet know; the DNA thus transferred may acquire incorporation into a chromosome-like body in order to replicate. But the fact that a genetical unit of the chromosome can be transmitted outside the cell prompts some reflection on the meaning of the classical conception of the cell as the irreducible unit of life. (See Baker (6) for a most scholarly discussion of the Cell Theory). It may be unsound to draw general conclusions from bacterial studies. The existence of chromosomes in bacteria is an open question, and the success of transfer experiments may very well be due to the primitive organization of the bacterial hereditary apparatus. The recent results on the artificial transfer of DNA between varieties of duck (8), should their validity be established, would, however, more than justify such reflections.

The above studies on extra-nuclear inheritance and extracellular transmission have laid emphasis on the autonomy of subcellular units and on the dissociability of genetic transmission from cellular reproduction. The net result of such emphasis is to direct our attention to a singular feature of the cell—its function as a limiting reproductive unit. The boundaries of a cell describe the reproductive limit of the living world. No subunit of a cell, be it parasitic or symbiotic, can reproduce its kind in the inanimate environment. Where this feature dominates our conception of cell organization, the mechanical unity of the cell appears more as a particular instance of organic association than as an ultimate unit of organic function. Units exist within units at all levels of the biological kingdom, and subunit autonomy is incorporated into unit integrity by relationships we have yet to explore. This is as true for the cell as it is for human society.

A limiting unit is, in the sense used here, an enclosure, and enclosures, be they of cellular or geographic proportion, share certain features of the evolutionary process. A species of cell perpetuates its type by retaining a constant intracellular population of self-duplicating elements, nuclear or cytoplasmic. In view of the functional autonomy of these elements, their capacity to maintain their respective repro-

ductive rates must be matched by the cell's capacity to limit them. It may be supposed that some form of competition for metabolic substrate exists between the different genetic elements of the cell, but, since their survival is tied to the cell as a whole, it must also be supposed that evolution has selected the appropriate mechanisms to maintain a steady state. It is only in unusual cases that this state may be permanently disturbed without erasing life. The loss of chloroplasts, blepharoplasts or oxidative particles are instances of such cases. In bacteria the possibilities of interfering with steady state mechanisms have been explored by Cohen (18). By use of inhibitors or by restriction of essential nutrients he has been able to produce "unbalanced" growth of nuclear versus cytoplasmic moieties. This, if uncorrected, leads eventually to death of the cells.

In considering the experimental data on mitotic metabolism it should be borne in mind that the classical mechanical picture of the cell is inadequate. Undoubtedly our newer ideas of machinery derived from electronics and expressed in terms of Information Theory will ultimately lead to a more precise understanding of biological mechanisms. But we must always deal with the fact that the cell is not only an evolved unit but also an evolutionary one. Mechanisms, as pointed out by Whitehead, are not identical with the relationships between mechanisms (112). And relationships at any instant are the fabric of evolution, even where evolution is infinitesimally slow. To understand the metabolic processes leading to and embracing the division of cells, the relationships between metabolic mechanisms are as important as the mechanisms themselves. We have as yet a very limited knowledge of these relationships but enough to recognize that few of the metabolic mechanisms required for mitosis are associated with it exclusively.

MITOTIC METABOLISM

From their beginning, metabolic studies of cell division had one common goal—to discover the unique factor initiating the process of mitosis. At the turn of the century investigators devoted much of their attention to changes in nucleocytoplasmic ratios during cell maturation in the hope that these would provide a basis for the shift from normal to mitotic metabolism. But, despite the many correlations between relative mass of cytoplasm and the initiation of

mitosis, the secondary and faculative nature of the relationship was clarified by biologists as outstanding as Conklin (19) and Bailey (4). During the twenties, interest was focussed on mitotic stimuli and respiratory cycles, but along these lines, too, no universal pattern was established (11). In the thirties, when biochemistry had acquired the full stamp of success, the objective was the sulfhydryl group (-SH) which, thanks to the school of F. G. Hopkins, had come to be regarded as a pivotal group in the control of cellular metabolism (44). Of all the metabolic approaches to mitosis, the last one has had the most enduring interest; but, like the others, its purpose was too broad in scope and its subject matter too narrow. Voegtlin and Chalkley (104) were the first to recognize that even so singular a metabolite as glutathione might be effective only at one phase of division, in their opinion, that of nuclear reproduction. However, the common practice was to treat the whole of cell division as a single process controlled by a single metabolic system.

Recently, because biochemical studies have concentrated on specific phases of the division cycle, there has been considerable advance in our understanding of mitotic metabolism. Chromosome duplication, the sources and nature of the simpler constituents of chromosomal substance, and the mechanics of chromosome separation have all been investigated in biochemical terms. To fit the many results into one metabolic scheme is possible only if we regard a collection of metabolic events, each germane to some phase or facet of mitosis, as an adequate scheme. But, in so defining mitotic metabolism, we relinquish the view enthusiastically held by many that a unified chemical theory of mitosis is a meaningful goal.

It is a fact well known to cytologists that chromosome duplication is not necessarily tied to mitosis. There are, in plant and animal tissues, many instances of polyploidy arising from the failure of mitosis to follow chromosomal replication (91). There are also a few instances of mitosis occurring in the absence of chromosome duplication (1). And, even where the two events are associated, as is most commonly the case, mitosis may or may not immediately follow chromosome doubling. In higher plants hormones may act on one without affecting the performance of the other (91), and in unicellular organisms non-optimal temperatures may inhibit cell division without influencing chromosome duplication (116). It may be concluded that the metabolic

conditions underlying chromosome reproduction are distinct from those underlying chromosome separation.

The immediate cause of chromosome duplication is unknown. There have been many attempts to find one, but such attempts have usually led to control mechanisms acting by stimulation or inhibition of whole metabolic systems. There are at least two ways by which chromosome duplication is controlled: limiting of metabolic supplies and hormonal stimulation of otherwise inactive metabolic pathways. Limitation of energy supply in some animal tissues (15), of sulfur in algae (38) and of thymidine in bacteria (18) are instances of the first kind. Hormonal control is more complicated. There is a qualitative factor which at least superficially is comparatively easy to assess; certain hormones like kinetin and indoleacetic acid affect some phase of mitosis, whereas others like gibberellic acid do not. But the regulatory power of hormones is also tied to quantitative factors which orient metabolic systems in one direction or another, according to the intracellular concentration of hormone (35, 107). Furthermore, different species of cells respond differently to the same hormonal concentration (76, 91). It may, of course, be that at the molecular level the effect of the hormone is fixed and specfiic, but such simplicity of reaction is bound to be lost if at the cellular level the molecular receiver of the stimulus is part of several subcellular systems. The broad effect of mitotic stimulants has been made particularly evident in studies of neoplastic growth. It might have been supposed that transformation of non-dividing or rarely dividing cells to rapidly dividing ones by what appeared to be a single principle involved a single triggering factor. Tumor-inducing bacteria, however, not only heighten the capacity of affected cells to produce kinetin and indoleacetic acid but also activate a variety of synthetic functions (12). The theoretical importance and practical utility of discoveries of specific mitotic stimulants is patent, but in the writer's opinion no generalization respecting the induction of mitosis will prove adequate if based on the action of such substances alone. The properties of mitosis need to be integrated at a more abstract level with at least three primary functions, namely, the specific action of mitotic agents, the potentials and limitations arising from cell differentiation, and the metabolic poise of the cell as a consequence of its physiological state.

A significant feature of chromosome duplication is that the terminal

phase of synthesis must occur in immediate association with pre-existing chromosomes. Two properties of this phase have become more or less apparent: the whole of the genetical structure of the chromosome is reproduced at one time, and the complex of biochemical reactions supporting the reproduction is not catalyzed by the genetic moiety of the parent chromosome.

The evidence for a simultaneous reproduction of genetic parts is to be found in the classic labelling experiments of Howard and Pelc (45) with bean root cells using P^{32} to mark nucleic acids and S^{35} to mark the protein. Histo-chemical measurements also indicate that histones, which quantitatively are the major protein constituent of chromosomes, are reproduced at about the same time as DNA (67, 100). Results by newer interferometric techniques point to a constancy of chromosome mass during mitosis (64), although it may be presumed from isotopic studies that following mitosis relatively little additional synthesis of chromosomal substances takes place.

More important, however, than the proportion of chromosomal substance formed at the time of replication is the genetic identity of the components reproduced. It is apparent from various lines of study that some chemical constituents of chromosomes fluctuate in the course of interphase metabolism and are therefore unlikely to be carriers of genetic pattern. Acidic or residual proteins (68) increase in concentration in proportion to cellular metabolic activity (41); they are not formed in appreciable amounts at the time of duplication and may be present in low concentration in proliferating cells (9). RNA is not only continually metabolized in the nucleolus (36) but also in the chromosomes. Although the latter is difficult to demonstrate directly, it may be presumed to be so from the fact that non-nucleolar RNA undergoes metabolic change in the nucleus (103) and that RNA is present in the chromosomes (77). DNA and histones, on the other hand, undergo little if any metabolic alteration in cells capable of multiplication (26, 67).

On the whole, the point of view commonly taken is that DNA is the seat of genetic pattern and that together with basic proteins (histones or protamines) it constitutes the essential structure of genetic transmission. The apparent absence of metabolic flux in these constituents is consistent with this view. The evidence, however, is not quite as unequivocal as opinion, or perhaps prejudice, would like

to have it. Reports persist on the lability of some chromosomal DNA (7, 69), to which, of course, two interpretations are possible: either genetic substance is subject to metabolic flux or genetic-like substance which fluctuates is non-genetic. This difficulty has not yet been resolved. It is the writer's opinion that whatever the findings on DNA lability, they are unlikely to alter markedly the conclusion that the genetic moiety of chromosomes is synthesized at one interval.

Successful replication requires only that new progeny be capable of further replication. For this reason the many changes which chromosomes undergo in the course of cellular differentiation or of interphase function are parenthetical to the story of genetic continuity. If the present outlook is correct—that genetic constituents are synthesized simultaneously and that those not so synthesized are secondary agents in duplication—then the genetic component of newly formed daughter chromosomes should at once be capable of further reproduction. Development, the cumulative change in property arising from the interaction of biological units with their environments, should not be part of the reproductive cycle of genetic elements. And if so, it follows that chromosome replication is controlled from the start by factors outside the genetic moiety of the chromosome. The intracellular location of such factors, their number and mode of operation, is an open question, but they must all share the property of somehow preventing continuous chromosomal replication.

Current opinion that the enzymes directly supporting chromosome reproduction are not part of the chromosome is based on the belief that specific macromolecular patterns are formed by mechanisms of code transmission not by a purely enzymatic weaving of polymers. Development of this view began with the resolution of nucleic acid structure as a polymer of four principal nucleotides, and with the speculation of Gamow (29) that such a structure is sufficient to code amino acid sequences in all species of protein. Experimental proof that nucleic acids can themselves transmit genetic information provided a strong impetus for investigating self-duplication. Crystallographic studies of DNA structure led to the Watson-Crick model of a double-stranded helix (110) which not only accounted for the physical and chemical data but also provided a molecular version of self-duplication in that a single helix of DNA could couple only with its exact complement. The correctness of the hypothesis that

DNA is a twin-stranded structure is supported by the autoradiographic studies of Taylor and his associates (101). They found that during mitosis the parent chromosome behaved as though it consisted of four longitudinal DNA strands, two new and two old. Daughter chromosomes appeared to consist of one new and one old strand, thus confirming the speculation of Watson and Crick that the twin-stranded structure would uncoil and a parent strand couple with a newly formed one. Although some investigators (53) have questioned the completeness of the autoradiographic evidence, there has been no contradiction of the essential principle involved.

Replication of a molecule by virtue of structural complementarity instead of by solely activating a complex of enzymes has established the importance of cybernetic principles *vis-a-vis* those of chemical catalysis in the interpretation of growth. Energy production and chemical transformations are regarded as the province of enzyme action, but the specific ordering of components is presumed to be mediated by code-transferring mechanisms. The "sense or nonsense" principle is the simplest mechanism of this type which functions by accepting or rejecting, according to suitability, the enzymatically activated molecules presented for polymer formation. Given a non-enzymatic process as the ultimate step in self-duplication, it is apparent that the genetic moiety of the chromosome need not have catalytic properties.

In both phage and plant virus infection the necessary and sufficient agent of genetic transmission is nucleic acid. Proof of this, though not absolute, is strongly convincing (see Section I). It is the events which follow infection by these agents that are not clearly defined. One unanswered question is whether genetic pattern is transferred directly from parent DNA to the growing polydeoxynucleotide chain or whether protein serves as an intermediate. The consensus is in favour of direct DNA transfer (20), but some studies of bacteriophage reproduction allow for the possibility that minutes after infection a temporary transfer of pattern occurs from DNA to a non-nucleic acid substance (21), presumably protein. Besides this particular question, there are many which follow from the fact that viral or chromosomal duplication is accompanied by the activation of a whole metabolic network. Such activation is made possible by the capacity of infective nucleic acids to order amino-acid sequences in proteins. It is, however, beyond the province of this review to discuss the field of relationships

between enzyme formation or activity and nucleic acids; it will be sufficient to assume that, given the genetic unit, specific enzyme complements are possible.

If the chromosome in a newly formed daughter cell is, by virtue of its genetic make-up, already capable of inducing formation of the necessary reproductive enzymes, it follows that the temporary or permanent absence of reproduction lies in factors outside the genes. Such factors could be anywhere in the cell, including the chromosomes. Their nature could be as varied as the intricacy of cell organization allows. Even if the chain of events from daughter chromosome to daughter chromosome were identical through the biological kingdom, each link would offer the cell a point for inhibition or one for stimulation. It is important, therefore, to distinguish the sequence of events from their inhibition or promotion and to allow that the latter is likely to be more varied than the former.

It has been frequently proposed that mitosis is restricted by limiting energy (15, 91). A restriction of this sort implies the existence of subcellular systems competing for energy, the mitotic one being either a less effective or more highly demanding competitor, therefore dependent upon a relatively large energy pool. Or, competition for available energy could be pictured in which hormones tilt the balance, now in favour of one, now in favour of another. So far as chromosome duplication goes, the effectiveness of energetic control would largely depend upon the proportion of cellular material tied to the chromosomes; the greater the proportion, the greater the demand on the energy resources of the cell.

Yeast furnishes a convenient model of a cell with a low nucleocytoplasmic ratio. In terms of protein, cells of the wheat embryo have ten times and calf thymus cells (which are 60 per cent nuclear) have 60 times the proportion of DNA. One gram of dry yeast *(Saccharomyces)* contains about 1.6 mg. DNA (75). Roughly calculated, for each 3.5 mg. of chromosomal nucleoprotein there would be 70 mg. of RNA, 580 mg. of protein, and 350 mg. of carbohydrates and fats (111). Thus, during the life cycle of a yeast cell (less than 2 hours under optimal conditions) only a little more than 1/300 of the protein formed would be required for the chromosomes. Against about 70 mg. of cytoplasmic nucleotides the newly formed chromosomes would utilize only 1.6 mg. of deoxynucleotides. In two hours a colony

of yeast cells consumes 480 mg. of dextrose in doubling its number, but only 0.2 mg. of it is used for production of the principal chromosomal sugar, deoxyribose (111). How much of the life span of a yeast cell is associated with DNA formation we do not know, but if the data on lily microspores are taken as a very rough guide, we can set the upper limit as 1/20 (24). Thus, if we suppose all the deoxyribose of the yeast cell to be synthesized within five minutes, it would still claim no more than one per cent of the carbohydrate concurrently metabolized.

Clearly in the case of yeast it is difficult to imagine that chromosome reproduction is limited by an intracellular competition for energy. By contrast, in meristematic tissues cells with high nucleocytoplasmic ratios would be expected to suffer an appreciable drain on their synthetic capacities in the course of chromosome formation. Using the pattern of yeast metabolism as a guide, such cells might divert about 50 per cent of their metabolized carbohydrate during the interval of DNA synthesis. It may seem curious that in mature cells where sources of energy are often available as stored carbohydrate, divisions are rare; whereas in the meristem where chromosomes compete for a large portion of available substrate, cells are continuously in division. The brake on chromosome multiplication in differentiated tissues or mature unicellular organisms would appear to reside elsewhere than in the limitations of energy supply.

The singular localization of deoxynucleotides in chromosomes somewhat simplifies the search for conditions leading to chromosome duplication. Since low molecular weight deoxyribosidic compounds are presumably destined to form chromosomes and no other structure, their presence in a cell may be considered as a fairly direct pointer to gene replication. Speculations have occasionally been advanced that RNA is transformed into DNA (13, 77), but the evidence for this is thin compared to that for the direct utilization of low molecular weight deoxynucleosides. The puzzling and perhaps critical question is whether formation of deoxyribosides, their energetic activation by conversion to triphosphates, and the operation of polymerizing enzymes are all simultaneously induced or whether one link in the enzymatic chain serves to control the effective action of the others.

Enzymes capable of synthesizing polyribonucleotides and polydeoxynucleotides from the diphosphates and triphosphates of the com-

ponent nucleotides have been identified and purified (33, 74, 51). It is not known whether the capacity of cells to perform these enzymatic syntheses varies in correspondence with the cycle of duplication. On the other hand, it is now amply clear that trace amounts of deoxyribosidic compounds may be present in non-proliferating tissues and that these compounds increase when proliferation occurs. It had been known for some time that polydeoxynucleotides are stored in the cytoplasm of frog's egg (43) but this might have appeared to be a special case of substrate storage in marine eggs until Schneider showed that deoxyribosides commonly occur in animal tissues (84). The amount is small in relation to DNA (in rat liver it is about 6 μg. of deoxyriboside per mg. of DNA), but there is both a quantitative and qualitative shift in the pool upon initiation of proliferation (85). Whereas deoxynucleosides predominate in non-proliferating tissues, the phosphorylated forms alone or in combination with other groups predominate in proliferating ones (87, 98). A spectacular demonstration of the close relationship between the formation of low molecular weight deoxyribosides and chromosome replication is to be found in developing anthers of *Lilium longiflorum*. In these there is a 25- to 50-fold rise in soluble deoxyribosides at the intervals of DNA synthesis preceding meiosis of the microsporocytes, mitosis of the microspores, and at the time of DNA doubling in the generative nucleus (28). The sharp but brief appearance of these compounds strongly suggests that one of the earlier steps in the sequence leading to chromosome duplication is an activation of deoxyriboside-producing enzymes.

The locus of production and the mechanism of formation of these compounds is not yet clear. From the standpoint of intracellular relationships there is no evidence to suggest that any of the molecular constituents are formed in the nucleus. All the nitrogenous bases with the exception of thymine are common to RNA and DNA, and thymine may be derived from the common constituent cytosine (31). Phosphorylation of deoxynucleotides to an energy-rich state may occur in mitochondria (39, 82). Only the origin of deoxyribose remains unresolved, and the evidence for a rise in sulfhydryl groups in cells undergoing mitosis (92) may serve as a means of reducing ribosides to deoxyribosides (32). Although it is attractive to suppose that such reduction occurs in the nucleus, there is no evidence bearing on

the point. The one enzyme, pyrophosphorylase, which is known to be localized in the nucleus (86) is of a type similar to that catalyzing the linking of deoxynucleotide triphosphates into the DNA chain (51).

The intracellular loci of reaction sites have an obvious meaning for unicellular organisms where all biosynthetic reactions occur within the compass of the individual cell. The meaning, however, is not nearly so obvious in the case of multicellular organisms. Although it may be presumed that under appropriate conditions most meristematic cells are capable of independent growth and division, it is apparent that such independence does not obtain within the organism. Hormones are commonly assigned the role of metabolic governors to account for organismic control of cellular function, but in the case of chromosome duplication the controls may not be purely hormonal. The production of deoxyribosidic compounds furnishes a relevant example.

Deoxyribosides are not always synthesized *de novo* nor are they always produced in the cells where they are utilized. In lily anthers the variations in DNA content with development are such that it wou!d be difficult to explain the pattern without supposing that DNA, or perhaps apparent 'DNA', derived from surrounding tissues served as a source of deoxynucleosides for DNA synthesis in the microsporocytes and microspores (28, 57). One interpretation is that deoxynucleosidic compounds are formed by a breakdown of tapetal DNA (57); evidence in the writer's laboratory, however, is not entirely consistent with this interpretation (28). Nevertheless, there is no question that DNA synthesis in developing pollen is supported by rich extrace!lular sources of deoxyriboside. Similarly, upon initiation of germination in corn seeds, deoxyribosides are formed in the endosperm and presumably serve as a source of those found in the growing roots (14).

MOVEMENT AND SEPARATION OF CHROMOSOMES

Chromosome separation has a distinctive physiological trait; its occurrence depends upon a transient metabolic poise and potential, which, if unused, is lost to the cell's morphogenetic history. Not only is a direct connection lacking between the processes surrounding chromosome duplication and those surrounding chromosome separation, but there is no compelling sequence of events which ultimately leads

from a doubling in chromosome number to mitosis. A sequence obviously exists, but where it fails the cell simply reorganizes in response to its doubled chromosome complement. The disruption of a single mitosis by colchicine is common knowledge. It is possible permanently to destroy the mitotic mechanism by irradiation and so to have cells of growing volume persistently increasing their chromosome number (81). In synchronizing divisions of Tetrahymena cultures by temperature mitosis is arrested but not chromosome duplication (83). Polyploidy in root cells arising from a natural failure of mitosis is a familiar occurrence (91).

There are two striking characteristics of mitosis — polarity and rhythm. The one has its seat in the physical organization of the mitotic apparatus which has long been studied and about which much information has been gathered (99). The other is a feature of biological organization which has in recent times attracted much attention but about which little is known. In a number of cases where inhibitory agents have been used—carbon monoxide (99), mercaptoethanol (61) —the rhythmicity of the affected processes underlying mitosis has become apparent.

The events germane to chromosome separation begin at prophase with contraction and thickening of chromosome filaments. It is now recognized, mainly as a result of phase contrast micrography and cinematography, that not only fibrillar contraction but also protoplasmic swelling and autonomous chromosome movements play a role in the subsequent process of chromosome separation (5, 10). The structures involved in mitotic movement are usually grouped under the title of "spindle body" or "mitotic apparatus", but the term is both a complex and variable designation. In many Protozoa it refers to a purely intranuclear system. In plants the spindle body is largely nuclear but also includes some cytoplasmic substance; in animal eggs it may represent about 95 per cent cytoplasmic and five per cent nuclear material. In a most general way the spindle body may be described as a volume of protoplasm free of most cytoplasmic particles, containing chromosomes with their associated structures, and characterized by oriented fibrillar elements (46, 78, 99). In plants the oriented elements are mainly of nuclear origin, but in animals cytoplasmic asters are present, which in sea urchin eggs account for some 90 per cent of the spindle body.

There are two questions to which we should like to address this review: What is the nature of the metabolic processes which lead to spindle polarization and chromosome orientation; and what metabolic systems support the consumation of mitosis. To neither of these questions are there adequate answers; we have at present only piecemeal information.

There is evidence from studies of sea urchin eggs that the proteins of the spindle body are not newly synthesized for mitosis but are derived from a pre-existing protein (59). In plants, nuclear proteins reach their maximum concentration shortly after telophase (34); there appears to be no pre-mitotic synthesis of nuclear protein, and, since the spindle body is largely nuclear, it may be supposed that, like the sea urchin egg, no net synthesis of protein accompanies spindle body formation. The polarization of the spindle body may not be the work of proteins alone but may result from their interaction with other substances (99). RNA and polysaccharides have been identified in the spindle body (42, 48, 88, 97) and they appear to be synthesized prior to spindle organization. There is no satisfactory evidence for their orienting ability, but the fact that some metabolic antagonists of RNA are effective inhibitors of spindle function (22, 46) supports the belief that at least this substance plays an active role in genesis of the mitotic apparatus. Results with ultraviolet micro-beam irradiation (30) point to the nucleolus as functionally involved in mitosis, and, since the nucleolus is a site of intense RNA metabolism, the role of RNA in chromosome separation is given additional emphasis. It is of considerable interest that UV-irradiation of non-nucleolar portions of a grass-hopper neuroblast serves only to delay mitosis, whereas irradiation of the nucleolus, without affecting DNA synthesis, permanently destroys the mitotic capacity of the cell. The idea that a nuclear organelle serves as a key factor in the extensive if transient reorganization of protoplasm is inviting, but more evidence would be needed to ascertain that the nucleolus directly mediates spindle organization.

The spindle body is but one facet of the structural arrangements underlying mitosis. The chromosomes, by virtue of their contraction, orientation and autonomous movements, are active agents of their own separation. Unlike the events of duplication, those of mitosis appear to involve progressive chemical as well as physical changes in

the properties of the chromosome. Too little is known about the composition of mitotic chromosomes to outline a funtional pattern of changes, even though there is much in the literature on histochemically detectable variations during mitosis. The obstacle to adequate knowledge is clear enough; a number of chromosome properties bearing on movement and orientation must depend on the nature of the chromosome-protoplasm interface. So small are the quantities of the components involved that, unless they have a unique composition, they are well beyond the reach of present chemical techniques. Histochemical methods have the distinct advantage of microscopic resolution, but they are usually limited in specificity and quantitative significance, especially the latter. It would be presumptuous to suppose that the results of histochemical methods permit one to sift primary from secondary changes and superficial from deep-seated ones. They are probably no more than coarse pointers to a set of intricate events. Nevertheless, even as such, they are of considerable value in visualizing the changes which chromosomes undergo in the course of movement and separation.

The possible role of surface charge as a critical factor in mitosis has been fully discussed by Anderson (3). As yet, there is no direct evidence for the operation of surface charges during mitosis, although it is improbable that such physical forces should not be involved. Chemically, there is some evidence for the presence of sulfhydryl and lipid at the chromosome surface. A variable but intense histochemical reaction for sulfhydryl groups at the surface of nucleolus and chromosomes has been reported by a number of investigators (47, 89, 115). Since the reversible sulfhydryl-disulfide transformation is an important and common biological mechanism for cross-linking protein chains and for effecting protein contractions (11, 40, 60), one possible role of such groups at the chromosome surface is obvious. There is, however, no marked concentration of sulfhydryl proteins in nucleus or nucleolus (94), and it is doubtful, therefore, that the nuclear apparatus preferentially metabolizes via sulfhydryl mechanisms. From the standpoint of mitosis the interesting point is that under defined conditions the potentialities of the sulfhydryl group are utilized in chromosome morphogenesis.

Lipids, too, appear to undergo cyclic changes in mitotic chromosomes (52). It is supposed from staining reactions with orange and aniline

blue that these changes occur in a phospholipid coat which surrounds nucleoprotein. It has also been proposed that the lipid is combined with nucleoprotein, the liponucleoprotein complex having a number of sulfhydryl groups (115). The significance of some of these histochemical observations is supported by the discovery that some of the soluble deoxyribosides present in proliferating tissues are combined with ethanolamine and choline (87, 98) in the same way as are ribosides in the pathway of phospholipid synthesis. Deoxyribosides may therefore be active in the formation of chromosomal lipid, and if so, it may be supposed that the metabolic network supporting DNA synthesis is also functional in structural modification of the chromosome. This possibility is also supported by the observation that in lily anthers the alcohol solubilities of deoxyribosidic compounds at meiosis and mitosis are different (28), the implication being that the difference in pairing properties of the chromatids is reflected in a correspondingly different spectrum of lipophily of the deoxyribosides.

METABOLIC SUPPORT OF MITOSIS

Discussions of mitotic metabolism have for a long time been clouded by attempts to see in gross metabolic patterns the causal agencies of mitosis. No topic in this field has excited more controversy than the relationship between respiration, glycolysis and the initiation of mitosis (see Ref. 99 for a balanced review and 106 for a vigorous and somewhat impassioned exchange of views). In the writer's opinion, it would be far more fruitful to keep the coarse labours of the cell, which supply energy and substrates for many subcellular functions, apart from the intricate balance of subcellular competitions and synergisms which form the basis of morphogenesis. There are, in effect, only two lines of study which have been aimed at resolving the metabolic requirements of mitosis—those concerned with the nature of energy sources and those concerned with the functions of sulfhydryls.

Commonly—though not universally—mitosis in aerobic organisms is oxygen-dependent. Generally, however, where an oxygen requirement exists, it is anticipated before the termination of prophase. Many aerobic cells which have entered into prophase complete their mitotic cycle in the absence of oxygen (15). Some cells require oxygen during part of prophase; others do not (5). Some otherwise aerobic cells may not even show a pre-prophase requirement for oxygen. It has,

indeed, been Warburg's contention that the cause of cancer lies in an impairment of the respiratory system on the grounds that such impairment inhibits differentiation but not mitosis (106). Many tumors are characterized by high glycolysis and low respiration. Although the anomalous pattern is more likely to be an effect, not a cause, of carcinogenesis, it is nevertheless true that in normally aerobic cells mitosis is one of the few major cellular functions which can occur, partially or entirely, in the absence of oxygen. The variable mitotic requirements for oxygen are not easily explained. The competitive demands of subcellular organelles on the energy pool is probably an important factor which would vary with cellular differentiation. In roots, for example, the region of cell division shows the lowest respiration, and that of cell elongation the highest (49).

To our knowledge, the benefit of respiration to mitotic movement is in the provision of energy-rich compounds such as adenosine triphosphate (ATP). But no direct line of transfer has yet been established between ATP produced in respiration and that consumed in chromosome separation. ATP can be produced glycolytically, and, since glycolysis operates during mitosis, it alone may provide the required energy (11). There is, however, little evidence that glycolytic mechanisms are activated during mitosis. Microspores of *Lilium longiflorum* have a very low capacity for catabolizing sugar by primary glycolytic enzymes during the mitotic interval, but a very high capacity at other times (71). Furthermore, there is as yet only limited evidence that ATP is actually consumed in chromosome movement. Cultured animal cells which have been killed by repeated extractions with glycerol contract on treatment with ATP. In those cellular residues which contain anaphase figures the contraction causes a movement of chromosome groups (42). Isolated spindle bodies of the sea urchin also respond to ATP treatment (59). This is the most direct evidence on the specific role of ATP in the mitotic process, but even so, its significance is limited by the fact that inorganic polyphosphates can replace ATP *in vitro* and that in actual mitosis chromosome separation is only partly a consequence of the contractile process demonstrated in these experiments.

Sulfhydryl groups have provided a basis for the liveliest speculations on the chemical nature of mitosis. The primary reason for the interest —and one generally established—is that in tissues or colonies of cells

undergoing proliferation the concentration of sulfhydryl groups is high (92). Furthermore, since the sulfhydryl (thiol) radical is known to have both metabolic and structural functions, it has been inviting to interpret this high concentration in terms of a single chemical theory covering metabolic requirements and structural changes. Hammett suggested that -SH functions in mitosis as a preferential activator of nuclear proteolysis leading to chromosome reproduction and separation (37). Rapkine speculated that protein denaturation, by exposing insoluble -SH groups, reduced a store of oxidized glutathione which in turn activated glycolytic enzymes and thus initiated cell division (11). Brachet pointed to disulfide linkages in protein as a chemical basis for spindle gelation, a view which has received much attention in Mazia's studies (11, 59). To the extent that these and other speculations consider thiol compounds to be important to mitosis, they are on reasonably firm ground. It is in the attempts to assign singular mitotic functions to the thiol radical that the thinness of the speculations becomes apparent. This is so because of the ubiquity of sulfhydryl groups in nearly all fundamental cellular processes. Few phases of growth are not in some way and to some degree affected by the activities of the thiol radical (58).

In cells which are about to enter mitosis there is an increase in the concentration of reactive thiol compounds (92). In some cases it may be shown that the increase is due to a *de novo* synthesis of glutathione (92). In others there may be a conversion of oxidized to reduced forms (58). Many sulfhydryl poisons inhibit cell division (11). Some, like chloracetophenone, if applied to cultured cells may inhibit spindle formation, implying a structural role for thiols in mitosis (46); usually, the poison appears to act on metabolic processes. The important role of the thiol radical in mitosis is indicated by the failure of synchronously growing *Chlorella* cells to divide if sulfur is omitted from the medium, even though the absence of other important inorganic nutrients—N, P, K—has no such effect (38). Studies of *Trillium* anthers during microsporocyte meiosis have shown that major changes in the concentration of both soluble and protein -SH occur during the interval of chromosome separation (93). Cytoplasmic division also involves the thiol radical. In certain strains of yeast the absence of an enzyme capable of reducing protein disulfide groups is

associated with an incapacity of such cells to undergo cytoplasmic divisions (72).

The evidence is thus unambiguous on the point that in diverse types of cells the chemical potentialities of the thiol radical are utilized to support mitosis. The evidence is also fairly clear that thiols are utilized in more than one phase of the cycle. Two possible roles of sulfhydryls have already been mentioned—reduction of ribosides to deoxyribosides and chromosome contraction. A highly probable role is participation in the gelation and solation of the mitotic apparatus. This has been demonstrated in sea urchin eggs by means of isolated mitotic spindles and by use of sulfhydryl poisons to prevent or delay mitosis in fertilized eggs (59, 60, 62). It is also evident from studies of isolated lily microspores that during the mitotic interval some tightly bound disulphide groups appear in the proteins (94).

The most general thing which may be said about thiols in mitosis is that in many cells a reservoir of soluble -SH exists or is formed in response to mitotic needs. The pool of sulfhydryl thus made available is utilized by specific mechanisms for specific needs. There appears to be no particular morphogenetic effect arising directly from high sulfhydryl levels. Indeed, even the simple postulate of Rapkine that increased soluble -SH activates glycolytic enzymes does not hold. In lily microspores, at least, glycolytic enzymes may rise in activity while soluble -SH remains low, and may fall in activity when soluble -SH rises (94). It can be concluded only that, while sulfhydryls may be limiting to mitosis, they, like many other cellular substances, are utilized by specific metabolic systems. A definitive list of the systems which draw upon the sulfhydryl pool cannot yet be made, but it would appear that changes in chromosome morphology, spindle formation, deoxyriboside synthesis and possibly energy storage (99) are important items. Beyond this list lie deeper questions as to how subcellular units compete, if they do, for the thiol pool and how, in the presence of an adequate supply, activation of the appropriate metabolic system proceeds in ordered cyclical fashion.

METABOLIC CONSEQUENCES OF MITOSIS

Reorganization of the interphase nucleus into a set of highly condensed and motile chromosomes deranges, to a variable extent, the activities of cytoplasmic components not directly involved in mitosis.

The derangement is of two sorts. One occurs during division and affects those components which depend closely upon the nucleus for maintenance of function. Mitochondria are probably in that category; chloroplasts possibly not. The second occurs at the close of division, when both nucleus and cytoplasm are affected by the novel ratios of cellular constituents. The freshly organized nuclei are unusually rich in genetic substance, and the newly formed daughter cells are unusually rich in nuclei.

The post-mitotic growth of the cell is a developmental process governed by requirements which probably have no direct relation to mitosis. Such growth is in a number of ways similar to that taking place in unicellular organisms following partial amputation of their cytoplasm. In both cases the objective appears to be a return to the steady state condition prevailing in the mature cell. Chloroplasts grow or multiply to their original form or number, and so do other cytoplasmic inclusions (79, 80, 91). Commonly, under conditions of optimal nutrition, the chromosome set limits the quantity of cytoplasmic aggregate. This has been demonstrated in yeasts where the amount of protein, ribonucleic acid and oxidative activity per cell is directly proportional to the degree of ploidy (75). But this relationship between size of chromosome set and growth of cytoplasm is not inviolate, for, experimentally, changes have been induced in the respective rates of growth of nucleus and cytoplasm (18).

Disorganization of the interphase nucleus, as might be expected, affects different cells to different degrees. The basis for this variability may be partially found in studies of enucleation. To the extent that a cell in mitosis and an enucleated cell lack an organized interphase nucleus, their respective physiologies may be considered to be parallel, though obviously the parallel is of limited application. Most Protozoa perish quickly after nuclear removal (2). Some may survive for several days. Among the Paramecia, only *P. bursaria,* which contains symbiotic algae, survives for some time (2). *Acetabularia,* which is rich in chloroplasts, continues to function for upwards of one month following enucleation (36). It thus appears that the products of nuclear activity, upon which the organized survival of the cytoplasm depends, are stored within the cytoplasm in varying degrees of excess. Nucleocytoplasmic ratio and species of cytoplasmic inclusions are two of the more obvious factors governing the magnitude of excess, but they

are not necessarily the decisive ones. Cells like *Acetabularia*, which have a low nucleocytoplasmic ratio, probably store a large supply of nuclear products in their cytoplasm and therefore respond slowly to nuclear removal. The persistence of nuclear metabolites in the cytoplasm of *Acetabularia* may be inferred from the capacity of enucleated individuals to regenerate partially their gene-controlled umbrella form (36).

The significant point to emerge from enucleation studies is that the interphase nucleus participates directly in a number of physiological functions of the cell and that when these functions fail, cytoplasmic disorganization results. By analogy it may be supposed that during mitosis, when the interphase nucleus disappears, these same functions suffer, though to a lesser degree. The truth of the analogy is supported by a number of observations, some of which are grouped in the following summary:

a) Intracellular movements: In many plant cells examined under the light microscope, cytoplasmic streaming has been observed to cease during mitosis (46). In cultured animal cells studied cinematographically, mitochondria fragment and lose their normal motility (17). In large cambial initials, on the other hand, mitosis of the comparatively small nucleus proceeds without interrupting normal movements in the cytoplasm (4).

b) Respiration: In anthers of liliaceous plants, a sharp drop in oxygen consumption has been observed during nuclear division of microsporocytes and microspores (24, 95). This has since been confirmed in suspensions of isolated microspores (70). A similarly low respiratory rate is found in the meristematic region of root tips (25). Synchronously dividing *Tetrahymena* cultures show an arrest in respiration at the instant of cell division (116). By contrast, marine eggs, probably because of their large mass of cytoplasm, show no such pattern of variation in respiratory behaviour (91).

c) Biosynthesis: During mitosis Amoebae suffer a decline in capacity to esterify extracellular inorganic phosphate (62). They also show no increase in protoplasmic mass, growth beginning only after completion of cytokenesis (79). Interferometric measurements of chromosome mass in mouse tissues likewise indicate a constancy of weight during the interval

from prophase to telophase (64). Synthesis of large molecules containing phosphorus or sulfur is also arrested during microspore mitosis (102).

All the above metabolic changes could result from temporary failure in aerobic energy-producing systems, as is observed in mitotic cells which have a decline in respiration and phosphate incorporation. The indifference of the mitotic process to the temporary curtailment of energy supply may be due, as Warburg has argued, to glycolysis being the sole source of energy for mitosis (106), or as Bullough has proposed, to aerobic formation of an energy reservoir prior to mitosis (15). Cellular activities not immediately associated with energy production might be directly affected by the absence of the interphase nucleus, but to the extent that such activities decline without a source of energy they would be difficult to identify. The fact that mitosis temporarily arrests the respiration of some cells is difficult to interpret in terms of known intracellular relationships. *In vitro,* mitochondria perform their respiratory functions unaffected by nuclei (96), and even enucleate fragments respire at a normal rate, although unaccompanied by energy-yielding phosphorylation (2). The apparent *in vivo* dependence is therefore puzzling unless one assumes that the disorganization of the interphase nucleus not only cuts off a supply of metabolite to the respiratory units (presumably mitochondria) but in some way positively arrests mitochondrial function.

The factors depressing metabolic activities of mitotic cells cannot all be explained by analogy with enucleation. Undoubtedly the behaviour of enucleated cells emphasizes the extent to which the cytoplasmic elements of a particular cell are metabolically autonomous, and stresses the importance of nucleo-cytoplasmic ratios in governing the intensity of departure from normal metabolic patterns. But there are other purely mitotic considerations. The spindle body, for example, increases in size during mitosis, and in some cases it may come to occupy a relatively large volume in the anaphase cell. On account of this, many cytoplasmic bodies are restricted to a narrow peripheral zone. Although there is no evidence on the point, this restriction might have an effect on their normal functions. More important, however, is the time factor in mitosis. Nuclei are only temporarily disorganized and the duration of mitotic intervals varies broadly. Other conditions being equal, the longer mitotic period

should produce the stronger metabolic derangements. Such relationships unfortunately are difficult to resolve. It is particularly important to remember that the data on metabolic consequences of mitosis are most easily gathered from cells which have long mitotic intervals. Whether such changes have not been found in rapidly dividing cells because they are difficult to detect experimentally, or because the cells do not undergo such extreme changes remains to be determined.

CONCLUSIONS

At the bottom of the information here assembled on mitotic metabolism lies the conception of a cell as an association of genetically and functionally autonomous units. These units are the self-duplicating systems of nucleus and cytoplasm. Whatever the range of their autonomy, they are all equally dependent upon cellular integrity because of the cell's singular biological role as the limiting unit of reproduction. Mitosis is a process evolved to meet the unique requirements of chromosomes for equivalent segregation, but, inasmuch as cell survival depends upon such segregation, non-chromosomal units must support its execution. There is neither reason to suppose nor evidence to indicate that at the appropriate instant the whole metabolic potential of a cell is directed towards consumation of mitosis. Some cellular metabolic systems are indifferent to the initiation and progress of the process; others are necessary for a supply of substrate and may inhibit or stimulate the mitotic cycle; still others function directly in the biosynthesis of chromosomal substance and in mitotic segregation.

For any particular cell, the pattern of mitotic metabolism is a product of two functions; one quantitative, the other qualitative. The quantitative function is an expression of the relative masses of cytoplasm, nucleus and mitotic apparatus. The smaller the ratio of cytoplasm to either of the others, the greater is the change of pattern from interphase to mitotic metabolism. The qualitative function, which expresses the nature of the change, appears to involve a shift in direction and intensity of normal pathways instead of an introduction of novel ones. Even production of deoxynucleosidic compounds is not entirely restricted to the mitotic interval.

The quantitative features of mitotic metabolism are readily expressed in general terms, but not so for the qualitative ones. Undoubtedly those processes in a cell which are directly involved in chromosome

biosynthesis or segregation have widespread distribution. But, so varied are the supporting metabolic processes that unless account is taken of the particular metabolic setting in which mitosis is occurring, no common pattern can be found. Mitosis, therefore, cannot be adequately described in the superficial terms of a catalogue of metabolic pathways. The net metabolism of a mitotic cell is a product of symbiosis and competition among subcellular structures, each of which, if endowed with genetic continuity, tends to grow and multiply unless limited by sister structures. It is well beyond this review to integrate the many variables of intracellular control mechanisms into a single formulation. If, however, this discussion has clarified the importance of specific cellular conditions in determining patterns of mitotic metabolism, it will have served its purpose.

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CYTOTAXONOMY OF NICOTIANA. II[1]

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INTRODUCTION

In the fourteen years intervening since CYTOTAXONOMY OF NICOTIANA. I. appeared (Goodspeed, 1945), articles bearing on this subject have been relatively few. However, this period has seen the publication of a major work on *Nicotiana,* a monograph on the genus (Goodspeed, 1954) which is the culmination of investigations covering almost fifty years, results of many of which were previously unpublished. The objectives of the monograph are: first, presentation of evidence, geographic, morphological and cytogenetic, contributing to interpretation of origins, evolution and relationships of the modern species of *Nicotiana;* second, application of this entire body of evidence to a revised taxonomic organization of the genus.

Since the monograph contains many data not elsewhere published, emphasizing the cytogenetic approach to taxonomy, it will be drawn upon extensively in the present review of the cytotaxonomy of *Nicotiana.* In order to avoid repetitious citation, it will be understood that statements not otherwise documented are referable to the monograph. For further detailed cytogenetic data and their interpretation, and for complete morphological and distributional data, the reader is referred to the monograph where some one hundred and twenty maps, drawings and charts supplement descriptive text.

The earliest *Nicotiana* investigations, begun in 1904 by the late Dr. W. A. Setchell, were concerned only with genetic analysis of varieties of *N. tabacum* and with such other species as were in American Indian cultivation, pre- and post-Columbian. As early as 1915, however, there was begun at the University of California Botanical Garden in Berkeley, a living collection of species of *Nicotiana* which ultimately included all but three of the currently recognized species, to permit morphological and cytogenetic investigations on an extremely extensive scale.

The majority of the species of *Nicotiana* are native to South Amer-

[1] Contribution No. 153 from the University of California Botanical Garden, Berkeley. Supplement to article in The Botanical Review 11:533-592. 1945.

ica. Three expeditions to South America before 1945 and three since that time not only determined the distribution and extent of natural variation of species native to that continent but were the means of bringing many new or previously little known species and varieties into the living collection at the Botanical Garden. Only two of the recognized South American species remain unknown in the living condition; only one of the fifteen species native to Australia and the South Pacific is lacking from the collection at the Botanical Garden, thanks to seed accessions received through the cooperation of colleagues in those areas.

TAXONOMY

The historical background of the taxonomy of *Nicotiana,* previously referred to briefly, is outlined in some detail in the monograph, Part VI (Goodspeed, Wheeler and Hutchison, 1954). Certain revisions in taxonomic organization have been made since 1945, and a summation of the current organization is included here:

Subgenus RUSTICA

Section PANICULATAE: *N. paniculata* L., *N. knightiana* Goodsp., *N. solanifolia* Walp., *N. benavidesii* Goodsp., *N. raimondii* Macbr., *N. cordifolia* Ph., *N. glauca* Grah.

Section THYRSIFLORAE: *N. thyrsiflora* Bitt. ex Goodsp.

Section RUSTICAE: *N. rustica* L. var. *pavonii* (Dunal) Goodsp., var. *pumila* Schrank, var. *brasilia* Schrank

Subgenus TABACUM

Section TOMENTOSAE: *N. tomentosa* R. & P., var. *leguiana* (Macbr.) Goodsp.; *N. tomentosiformis* Goodsp., *N. otophora* Griseb., *N. setchellii* Goodsp., *N. glutinosa* L.

Section GENUINAE: *N. tabacum* L.

Subgenus PETUNIODES

Section UNDULATAE: *N. undulata* R. & P., *N. wigandioides* Koch & Fint., *N. arentsii* Goodsp.

Section TRIGONOPHYLLAE: *N. trigonophylla* Dun., *N. palmeri* Gray

Section ALATAE: *N. alata* Link & Otto, *N. langsdorffii* Weinm., *N. bonariensis* Lehm., *N. forgetiana* Hort. ex Hemsley, *N. longiflora* Cav., *N. plumbaginifolia* Viv., *N. sylvestris* Speg. & Comes

Section REPANDAE: *N. repanda* Willd., *N. stocktonii* Brandeg., *N. nesophila* Johnst.

Section NOCTIFLORAE: *N. noctiflora Hook.*, var. *albiflora* Comes; *N. petunioides* (Griseb.) Millán, *N. ameghinoi* Speg., *N. acaulis* Speg.

Section ACUMINATAE: *N. acuminata* (Grah.) Hook., var. *multiflora* (Ph.) Reiche, var. *compacta* Goodsp.; *N. pauciflora* Remy, *N. attenuata* Torr., *N. longibracteata* Ph., *N. corymbosa* Remy, var. *australis* Goodsp.; *N. miersii* Remy, var. *lychnoides* (Remy) Goodsp.; *N. linearis* Ph., *N. spegazzinii* Millán

Section BIGELOVIANAE: *N. bigelovii* (Torr.) Wats., var. *wallacei* Gray, var. *quadrivalvis* (Pursh) East, var. *multivalvis* (Lindl.) East; *N. clevelandii* Gray

Section NUDICAULES: *N. nudicaulis* Wats.

Section SUAVEOLENTES: *N. suaveolens* Lehm., *N. maritima* Wheeler, *N. velutina* Wheeler, *N. gossei* Domin, *N. excelsior* Black, *N. megalosiphon* Heurck & Müll. Arg., *N. exigua* Wheeler, *N. goodspeedii,* Wheeler, *N. ingulba* Black, *N. stenocarpa* Wheeler, *N. occidentalis* Wheeler, *N. rotundifolia* Lindl., *N. debneyi* Domin, *N. benthamiana* Domin, *N. fragrans* Hook., var. *fatuhivensis* (Brown) Goodsp.

Many of the species above listed have been otherwise designated by various investigators over the years. For example, until relatively recently, *N. petunioides* (Griseb.) Millán was known as *N. pampasana* O. Kuntze, *N. pauciflora* Remy as *N. caudigera* Ph., and *N. noctiflora* Hook. as *N. cavanillesii* Dun. (For complete synonymy see Goodspeed, Wheeler and Hutchison, 1954.)

Comparison of the above with the earlier taxonomic arrangement (Goodspeed, 1945) will show the addition of three sections, REPANDAE, BIGELOVIANAE and NUDICAULES, composed of 24-paired species which were previously included in sections ALATAE and ACUMINATAE. This reorganization recognizes the union in amphiploidy of 12-paired progenitors possessing morphological characters of two distinct sections. Thus, species of section REPANDAE incorporate certain characters of members of section ALATAE and others of members of section TRIGONOPHYLLAE; species of section BIGELOVIANAE show some resemblances to members of section ALATAE, others to members of section ACUMINATAE; the single species of section NUDICAULES

appears equally related to members of sections ACUMINATAE and TRIGONOPHYLLAE. These morphological reflections of relationship have been found to be fully supported by cytogenetic evidence, and the combined evidence thus justifies sectional recognition of these amphiploids corresponding to that long given *N. rustica* and *N. tabacum*. On the other hand, *N. arentsii* has been retained in section UNDULATAE because its affinities are entirely intrasectional, both of its 12-paired progenitors being members of that same section.

Goodspeed (1947a) recognized and commented upon five definite but interrelated clusters or "nuclei" of species, which he termed "rusticoid", "tomentosoid", "alatoid", "noctifloroid" and "corymbosoid", and showed that these represent the basic framework of the genus as contrasted with species and groups of species which combine elements of these basic groups and transform the evolutionary pattern of the genus from a strictly dendroid one to a reticulate one. Later (Goodspeed, 1953) these informal designations, together with those referring to "genetic groups", were abandoned in this sense, and the use of "oid" as a suffix attached to a sectional name was retained only to refer to ancestry.

Clausen (1949) proposed an organization of *Nicotiana* which subdivided the eleven then-recognized sections into twenty-four "primary cytogenetic classes" to which he referred loosely, for convenience, as "subsections" without, however, ascribing to them taxonomic status. This recognition of "subsections" corresponded in many instances with the earlier recognition of "groups" within sections and of the outlying character of certain species in relation to others in the same section (Goodspeed, 1945). It appears that subsectional grouping should not be translated taxonomically because consideration of all evidence available points to the fundamental relationship of outlying species to others of their respective sections, despite the hybridization which has obviously entered into their evolution (Goodspeed, 1953).

Clausen (*l.c.*) questioned the validity of specific distinction between *N. trigonophylla* and *N. palmeri; N. longiflora* and *N. plumbaginifolia; N. bigelovii* and *N. clevelandii; N. stocktonii, N. nesophila* and *N. repanda*. Goodspeed (*l.c.*) also pointed out the close relationship of these species and an even closer morphological relationship between *N. corymbosa* and *N. linearis* and between *N. paniculata* and *N.*

knightiana, but considered that all evidence at present available justifies their specific separation.

Two species not in the previous taxonomic listing are shown here: *N. forgetiana* is now recognized as a member of section ALATAE, *N. longibracteata* as a member of section ACUMINATAE.

As seen in the above taxonomic arrangement, varieties have been designated in a number of species: *N. rustica, N. tomentosa, N. noctiflora, N. acuminata, N. miersii, N. corymbosa, N. bigelovii* and *N. fragrans.*

In the taxonomic organization (Goodspeed, Wheeler and Hutchison, 1954) in the monograph the genus was not typified, although *N. tabacum* L. has frequently been mentioned as lectotype. Recognition of such typification would necessitate certain nomenclatural alterations. Thus, subgenus TABACUM (Don) Goodsp. would become subgenus NICOTIANA, and section GENUINAE Goodsp., section NICOTIANA. In addition, it is clear that section RUSTICAE Goodsp. should become section RUSTICA, since it contains the type of subgenus RUSTICA (Don) Goodsp. (*N. rustica* L.). Section ACUMINATAE Goodsp., containing *N. acuminata* (Grah.) Hook., the type of subgenus PETUNIOIDES (Don) Goodsp., should become section PETUNIOIDES. These revised designations will not be applied in the present discussion for convenience of comparison between it and the publications here reviewed where the unrevised designations are employed.

In what follows, the relation of the cytogenetic evidence to the validity of the above taxonomic organization of the genus will be emphasized. It is to be borne in mind, however, that the taxonomic organization represents a product not only of the cytogenetic evidence but also of that from distribution and morphology.

SPECIES CYTOLOGY

In the Solanaceae approximately two-thirds of the 260 species for which chromosome number is known show 12 or a a multiple of 12 as the haploid number. In the related family, Scrophulariaceae, approximately one-fourth of the species show 6, 12 or 24 pairs.

Of genera of the Solanaceae most closely related to *Nicotiana, Petunia* shows 7 or 9 pairs of chromosomes, *Cestrum* 8 and *Vestia* 8. In *Nicotiana* known haploid numbers are 9, 10, 12, 16, 18, 19, 20, 21, 22 and 24. Twenty-eight species possess 12 pairs and eleven, 24 pairs—over

two-thirds of the total number of species for which chromosome is known (57 species). Thus, 12 or a multiple of 12 is the modal chromosome number in the Solanaceae and in the genus *Nicotiana.*

Within the genus there is an obvious correlation between chromosome number and taxonomic organization: subgenera RUSTICA and TABACUM contain only 12- and 24-paired species; subgenus PETUNIOIDES contains, in addition to 12- and 24-paired species, two aneuploid sequences, one below 12 (9- and 10-paired species) and the other below 24 (16-, 18-, 19-, 20-, 21-, and 22-paired species), each sequence confined to a single section.

While many species of *Nicotiana* exhibit polymorphism, some rather strikingly, no species is polynumeral. In other words, chromosome number is constant in varieties and races of each of the fifty-seven species so far as they have been examined. For example, early investigators found that numerous varieties of *N. rustica* all showed 24 pairs. Similarly in *N. tabacum,* despite its extreme variability and wide geographical range, recent studies of fifty-five races (Cameron, 1952) and of eight races (Murty and Swaminathan, 1956) revealed no deviation from 24 pairs of chromosomes. Recently Ahuja (1955) has reported $n=24$ for *N.* "companulata" H.B.K. and for *N.* "sanguinea" Link & Otto, two races of *N. tabacum.*

Although the occurrence in nature of any variation in specific chromosome number is unknown, haploidy, euploidy and aneuploidy are readily induced and have occasionally occurred spontaneously in cultivation. Incidence of these states is known in species of lower and of higher chromosome number and in those of every section of the genus (cf. Goodspeed, 1954, Ch. 10). Haploidy is of most frequent occurrence in F_1 interspecific hybrid populations, following parthenogenetic development in certain embryo sacs induced by germination of foreign pollen on the stigma or through stimulation by growth-promoting substances liberated by the effective fertilization in other embryo sacs in the ovary. Haploid androgenesis is less common. Haploid plants of the pollen parents have been reported in the following F_1 populations: *"N. digluta"* x *N. tabacum* and *N. glutinosa* x *N. repanda* (Kehr, 1951); *N. glutinosa* x *N. sylvestris* (Takenaka, 1952); *N. tabacum* (3*n*) x *N. langsdorffii* and *(N. tabacum* x *N. sylvestris)* x *N. sylvestris* (Kostoff, 1943).

At least twenty normally diploid species of *Nicotiana* are known in

the tetraploid state, some of them occurring in x-ray progenies, but the majority of them colchicine-induced. Fardy and Hitier (1945) made comparative studies of fifteen species in the tetraploid and diploid conditions, in terms of meiotic behavior and fertility.

In *N. tabacum* all twenty-four $2n$—1 types are known (Clausen and Cameron, 1944); in *N. sylvestris* all twelve $2n+1$ types (Goodspeed and Avery, 1939) and in *N. langsdorffii* all nine $2n+1$ types have been obtained (cf. Abraham, 1947). The majority of the trisomics of *N. sylvestris* and those of *N. langsdorffii* studied were distinctive in external morphology from the normal diploid and from one another. Similarly, the twenty-four monosomic types of *N. tabacum* differed from the diploid and from one another in a number of morphological characters.

Although no 6-paired species of *Nicotiana* is known, and reports of 6 in other genera of the Solanaceae have never been confirmed, there is evidence supporting the postulate that 6 is the basic chromosome number for *Nicotiana,* and that both 12 and 24 are derived numbers. Supporting this postulate is the predominance of 12-paired species and their compound morphological character, and the frequency of 4 to 8 pairing with a mode of 6 pairs in a large number of F_1 hybrids combining 12-paired species. Additional support is believed by some investigators to be provided by secondary association and pairing in haploids of 12-paired species (Kostoff, 1943). Takenaka (1951) observed secondary association of bivalents in diploid *N. glutinosa;* he also reported multivalent formation at the first meiotic metaphase in *N. nudicaulis* (1953a) and in *N. langsdorffii* (1956b). The latter, together with Kostoff's observation (*l.c.*) of secondary association and some bivalents in haploid *N. langsdorffii* was interpreted by Takenaka (*l.c.*) as evidence that the basic chromosome number in the genus is certainly lower than 9. Occurrence of chromosome associations higher than quadrivalents at first meiotic metaphase in autotetraploids of certain 12-paired species (Fardy and Hitier, 1945) suggests the compound character of those 12-paired species.

The known origin of present-day 24-paired species in amphiploidy between progenitors of specific modern 12-paired species, together with the suggestion that in the past 6-paired species existed, has led to the postulate that the present 12-paired species are of similar origin (Goodspeed, 1954). Thus, in what follows reference will be made

to 6-, 12- and 24-paired levels as basic elements of the evolutionary picture.

Chromosome morphology in fifty-six of the fifty-seven[1] species of *Nicotiana* known in the living condititon has been determined. The possession by members of subgenus RUSTICA of almost exclusively median or submedian centromeres, and by members of subgenus PETUNIOIDES of almost as many chromosomes with subterminal as with median or submedian centromeres may be significant in view of other evidence that among members of subgenus RUSTICA are the most primitive species of the genus while subgenus PETUNIOIDES contains the more advanced species.

In some species of *Nicotiana,* at least, the number of nucleoli is a relatively constant character. Studies of the relation between the number of satellites and the number of nucleoli in thirty-nine species (Goodspeed, *l.c.*) in general support the point of view that in most genera nucleolus-organizers are associated with satellites. In some species of *Nicotiana,* however, more nucleoli than satellites are present, nucleoli apparently being produced at nucleolus-organizing areas other than those associated with satellites. Some other species show fewer nucleoli than number of satellites, presumably the result of loss or functional failure of certain nucleolus-organizers. Thus, in *N. rustica* eight nucleoli were reported although only two satellited chromosomes were observed, while in *N. tabacum,* four nucleoli and six satellited chromosomes were reported. By contrast, Izquierdo and Duran (1957) found in the diploid race of *N. rustica* which they studied a maximum of five nucleoli, and in the tetraploid, a maximum of twelve nucleoli; in *N. tabacum* $2n$ they found three nucleoli and in $4n$, five nucleoli.

Studies of chromosome morphology in numerous varieties, forms and races of many of the more polymorphic species of *Nicotiana* indicate that there is no corresponding visible intraspecific chromosomal polymorphy. Nevertheless, there is abundant evidence that structural chromosomal distinctions between varieties do exist. *N. tabacum,* because of its wide distribution, extreme polymorphy, and the availability of numerous commercial and non-commercial races, has been the

[1] *N. ingulba* Black has recently been grown at the Botanical Garden in Berkeley from seed from Alice Springs, N. T., Australia, supplied by R. A. Steinberg of the United States Department of Agriculture, Beltsville, Maryland. Chromosome counts have been made here in first division of the nucleus in microspores of this species and the number found to be $n=20$.

subject of extensive cytological and genetic investigation. Structural distinctions between the tomentosoid subgenom in *N. tabacum* "Machu Picchu" and that subgenom in *N. tabacum* "Purpurea" were shown to exist by comparison of cytological behavior in the two F_1 hybrids combining each of those two races with the same species, *N. otophora* (Goodspeed, 1954). Cameron (1952) studied structural relationships of fifty-five races of *N. tabacum* by means of hybrid analysis and found that twenty-five races with large seeds were structurally identical with *N. tabacum* "Purpurea" while twenty-nine races with small seeds and weedy growth habit differed from "Purpurea" by one to several reciprocal translocations (cf. also, Mallah, 1943, 1952; Moffet, 1945). Murty and Swaminathan (1956) observed meiotic irregularities in three of the eight races of *N. tabacum* which they studied. They also analyzed meiotic behavior in F_1 hybrids combining six different parent races from widely different geographic sources, one of them combining two large-seeded races. On the basis of observed meiotic irregularities in all three hybrids, these investigators concluded that, while structural chromosome alterations, in addition to gene mutations, have played a significant part in the differentiation of races of *N. tabacum,* no correlation could be established between seed size or geographic distribution and such chromosome alterations. They also discussed the significance of the presence of structural chromosome differences in races of *N. tabacum* with reference to practical breeding in terms of inheritance, via linkage relationships, of blocks of deleterious characters along with desirable characters.

Despite the interspecific chromosomal distinctions which have been demonstrated to be present in *N. tabacum,* no identifiable differences exist in visible chromosome morphology. Indeed, interspecific differences in chromosome morphology are slight or lacking between certain closely related pairs of species (e.g., *N. paniculata* and *N. knightiana; N. palmeri* and *N. trigonophylla; N. longiflora* and *N. plumbaginifolia; N. nesophila* and *N. stocktonii).* Thus, the structural chromosome distinctions which have doubtless occurred, along with genic ones, in the evolution of these pairs of species have not visibly affected their chromosome morphology.

Intraspecific karyotypic constancy and the basic equivalence of karyotypes of closely related species, together with chromosomal distinctions which identify species that are taxonomically more remotely

related, give to chromosome morphology at least limited phyletic significance.

Since 1945, meiotic chromosome behavior in species of *Nicotiana* has been rather intensively investigated, in both mega- and microsporogenesis (Goodspeed, 1946, 1954), with particular emphasis on the latter. Because of the significance attached to pairing in the first meiotic division in F_1 interspecific hybrids as evidence of species origins and relationships, and because knowledge of meiotic chromosome behavior in parental species is indispensible for interpretation of that behavior in hybrids, a resumé of those studies is included here.

The entire meiotic sequence has been analyzed in pollen mother cells of a series of species, species which are members of different taxonomic subdivisions and are distinguished from one another in chromosome number and/or morphology. Thus, meiotic prophases have been investigated in *N. glauca* (section PANICULATAE, subgenus RUSTICA), $n=12$, in *N. otophora* (section TOMENTOSAE, subgenus TABACUM), $n=12$, in *N. langsdorffii*, $n=9$, and in *N. longiflora*, $n=10$, (both species of section ALATAE, subgenus PETUNIOIDES). In *N. otophora* and in *N. langsdorffii* the morphology of individual chromosomes is sufficiently distinctive that identification of certain of them is possible at pachytene and later. The meiotic sequence from midleptotene to the production of the quartet of microspores has been fully described and illustrated (Goodspeed, 1954).

The first meiotic metaphase has been studied in pollen mother cells in thirteen species by Takenaka (1953a, 1956b) and by Goodspeed (*l.c.*) in many species selected from various taxonomic subdivisions —from all the subgenera and from the majority of the sections. Comparative studies have shown conspicuous similarity of certain bivalent configurations in the 24-paired amphiploid species with those in the related 12-paired species, thus supporting phyletic conclusions based on other evidence.

As a background to the cytology of reproduction in the genus, analysis has been made of the complete sequence of megasporo- and megagametogenesis in *N. tabacum* and of certain stages in *N. alata, N. rustica* and *N. rotundifolia.* In addition, the development, morphology and behavior of the male gametes in *N. longiflora* and other species have been analyzed (Goodspeed, 1947b). So far as observed, embryo sac development in the genus is of the monosporic type. Dis-

tinctions in the shape of the embryo sac to be seen in *N. tabacum, N. rustica* and *N. rotundifolia,* each species from a different subgenus, are of interest and may or may not have phylogenetic significance.

Twin megaspore mother cells, each cell with a separate nucellus, are not uncommon in some species of *Nicotiana.* Cameron (1949) reported multiple seedlings in *N. tabacum*—of highest frequency in monosomic cultures—the majority of which were attributed to fertilization of two or more nuclei in a single embryo sac.

Distinctions in nucellar development and degeneration during megasporogenesis in species and in F_1 interspecific hybrids have been noted (Goodspeed, 1946; Brink and Cooper, 1947).

Normally, in those species of *Nicotiana* which have been investigated, the 8-nucleate embryo sac becomes a 7-celled megagametophyte, the differentiation of synergids, egg and antipodals being accompanied by wall formation (Bradley, 1948). Polar fusion is usually complete or nearly complete before fertilization. Among abnormalities noted in one race of *N. tabacum* is an increase in the number of nuclei in the mature embryo sac. From nine to sixteen nuclei have been observed following a fourth division of one to all eight of the nuclei of the mature sac. Another abnormality in the same race is the frequent migration of antipodals toward the micropylar end of the sac.

In the development of the male gametophyte the first mitosis occurs centrally in the microspore to produce the vegetative and generative nuclei; division of the generative nucleus normally occurs in the pollen tube in the upper third of the style. In those species studied, the male gametes undergo little structural alteration from the time of their origin to just prior to fertilization. In the tube and in the cytoplasm of the ruptured tube in the sac, they are slightly elongated, ovoid bodies (Goodspeed, 1947b; Bradley, 1948), each appearing to be surrounded by a cytoplasmic sheath. Just before fertilization the male gametes become more spherical; when in contact with the female nuclei, they expand and become more diffuse as they flatten against the nuclear membranes and gradually merge with the female nuclei. Contact of one male gamete with the egg nucleus usually precedes that of the other with the fusion nucleus; in the latter, however, the fertilization process is more rapid than in the former, and frequently two or more divisions have occurred in the endosperm before the first division of the zygote nucleus.

APOMIXIS

Reports of parthenogenesis in species of *Nicotiana* following emasculation and the frequent occurrence of maternals in artificially produced F_1 interspecific hybrid populations have long given evidence of at least some degree of apomixis in the genus (cf. East, 1930). Over a period of fifteen years, in investigations carried on in the University of California Botanical Garden, among six hundred and eighty-two interspecific crosses in which some viable seed was obtained, approximately 17% gave maternals only or a mixture of maternals and a small number of hybrids (Goodspeed, 1954). In these hybridizations, maternals were produced by twenty-six species, representing every section of the genus. The fact that among these species are the majority of the 24-paired species, long recognized as amphiploids, may possibly give some indication that in *Nicotiana,* as in other genera, there is a certain association of apomixis with polyploidy (Löve and Löve, 1949). From the point of view that the so-called diploid species of *Nicotiana,* 12-paired, are of amphiploid origin from a former 6-paired level, the relatively high incidence of maternals in attempted hybridizations involving 12-paired species as the female parent does not weaken this postulate that apomixis is associated with polyploidy. At the same time, however, it indicates that level of polyploidy is not of fundamental significance and, indeed, suggests that hybridization itself, with or without chromosome doubling, may be the source of apomixis in the genus, providing as it does for the combination of genes necessary to promote the initiation of an apomictic cycle (cf. Stebbins, 1950).

Takenaka (1952) has reported apomixis in an attempted cross between *N. glutinosa* and *N. sylvestris.* In the thirty-four capsules produced, most of the seeds were abortive; only twenty-two plants were obtained, twenty of which proved to be *N. glutinosa,* one *N. sylvestris,* and one apparently a hybrid. Takenaka assumed that the twenty maternals had their source in chromosome doubling in the *N. glutinosa* egg cell or in fusion of the egg with a synergid, either process activated by hormones of the foreign pollen. The one *N. sylvestris* plant was assumed to have been produced androgenetically by fusion of two male gametic nuclei.

Kordjum (1955) found that several varieties of *N. rustica* crossed with pollen of distantly related species, alone or mixed with self

pollen, gave progenies resembling the respective seed parent, and in one case, at least, possessing the diploid chromosome number. Embryological observations showed that the pollen tubes reached the embryo sacs but that the two male gametes, visible in each tube, were not discharged into the embryo sacs. Concurrently, the beginning of endosperm formation and the inception of parthenogenetic development of the egg cell were observed.

Recent studies of apomixis in *Nicotiana* at the University of California Botanical Garden (Goodspeed, unpbl.) have been limited to three selected 24-paired species, *N. tabacum, N. rustica* and *N. nudicaulis,* all known to produce diploid maternals following attempted interspecific hybridization.

In *N. tabacum* no viable seed was produced after emasculation alone or when it was followed by pollination with pollen of distantly related species. However, in *N. rustica,* approximately twenty-five of six hundred and twenty-five flowers emasculated produced capsules with from one to twenty seeds each, which gave diploid *N. rustica* plants. After crossing with pollen of distantly related species a considerably higher percentage of capsules matured (approximately fifty in six hundred crossings), each containing a few seeds which gave rise to diploid plants of *N. rustica.*

Thus, *N. rustica* at least, and presumably certain other species also, contains genes providing the conditions necessary for apomictic reproduction, and such potential apomixis could obviously come to have taxonomic and evolutionary consequences of far-reaching significance.

F_1 HYBRID CYTOLOGY

In *Nicotiana*, data from meiotic behavior in F_1 interspecific hybrids are of particular significance for phyletic conclusions because of the great number of such hybrids in relation to the number of species and because these hybrids include the majority of the interspecific combinations which are essential for interpretation of relationships, intra- and intersectional as well as intersubgeneric. Compatibility permitting successful hybridization, with attainment of maturity, between species which are taxonomically so remote as to be members of different subgenera, is apparently more extensive in *Nicotiana* than in most other angiosperm genera. Approximately 35% of the two hundred

and twenty F_1 hybrids of *Nicotiana* which have been analyzed cytologically are intersubgeneric combinations, and approximately as many additional ones are intersectional within a subgenus. A corollary to this statement is the fact that in F_1 hybrids between species of *Nicotiana*, 65% of those analyzed show a modal number of pollen mother cells with less than complete pairing. This apparently means that in *Nicotiana* genic and structural chromosome alterations expressed in pairing failure and in species differentiation have evolved at a different rate than have those which have impaired compatibility as measured by successful hybridization.

As previously reviewed (Goodspeed, 1945) and more recently reported in detail (Goodspeed, 1954), distinctions in the amount of pairing at first meiotic metaphase in two hundred and fifteen F_1 interspecific hybrids of *Nicotiana* have led to the definition of five rather distinct pairing categories. Thus, "minimum" pairing, range zero to 4 pairs, mode zero, characterizes seventy F_1 hybrids; "low variable" pairing, range zero to 8 pairs, mode 2 or 3 pairs, is found in thirty hybrids; "high variable" pairing, range 1 or 2 pairs to almost complete pairing, with a mode at approximately one-half the number which would represent complete pairing in the hybrid involved, is to be seen in thirty-five hybrids. "Drosera scheme" pairing is characteristic of fifty hybrids combining species of unlike chromosome number, where pairing in the modal number of pollen mother cells corresponds to the chromosome number of the lower numbered parental species; "complete" pairing characterizes some thirty hybrids analyzed.

Recently (Goodspeed, 1946, 1954), prophase studies in representative hybrids in each of the five categories have demonstrated a correspondence between the extent of pachytene pairing and the extent of pairing at first meiotic metaphase and so have reaffirmed the significance of the latter as a criterion of relationships. In other words, metaphase pairing in the first meiotic division in an F_1 hybrid of *Nicotiana* is shown to be significant evidence of relationship of the two species combined because it is a reflection of a corresponding degree of pairing at pachytene, which in turn reflects the extent to which the chromosomes of the two parental species possess genic and structural homologies.

Complete pairing and "Drosera scheme" pairing obviously indicate fundamental similarity in the genic constitution of the parental genoms.

Minimum pairing, earlier misnamed "lack of pairing" (Goodspeed, 1945, 1946, 1947a), and low and high variable pairing indicate the presence of a number of genic blocks in common in the two species in each case, corresponding to the maximum number of pairs, the variability in number of pairs being due to the small size of many of these blocks. Such pairing provides evidence of chromosome reorganization as a factor in the evolution of the species concerned. It is assumed (Goodspeed, 1954) that maximum pairing in each case is consistently between the same chromosomes rather than reflecting random association where all chromosomes possess the same potentialities for pairing, and this assumption is to a degree borne out by the observed morphological equivalence of bivalents from one pollen mother cell to another and by the fact that in a statistically significant number of counts the mode and the maximum remain constant.

CYTOTAXONOMY

As already noted and as will appear in the chart (Fig. 1), the genus *Petunia* is taxonomically closely related to *Nicotiana.* A certain confirmation of this conclusion has been reported by Pogliaga (1952) who produced a hybrid between *Nicotiana tabacum* L. and *Petunia parodii* Steere. His description of the morphology of this hybrid indicates a dominance of *Nicotiana* characters and notes especially resemblances to *N. sylvestris* and *N. longiflora.* Such resemblances are of interest and should not be unexpected because of a) the participation of an early race of *N. sylvestris* in the amphiploid origin of *N. tabacum* and b) the postulated origin of section ALATAE (including *N. sylvestris* and *N. longiflora)* in the petunioid complex (cf. Fig. 1). Poliaga made cytological studies in pollen mother cells of the intergeneric hybrid and reported counts of 31 chromosomes (*N. tabacum* $n=24$, *P. parodii* $n=7$) including an occasional bivalent which he interpreted as an autosyndetic pair of *N. tabacum.*

The F_1 hybrids which had at that time been analyzed cytologically were listed in the previous review and in the monograph; those lists will not be repeated here. Takenaka has recently reported the amount of pairing in certain of these same hybrids and in five additional F_1 hybrids not earlier reported. It will be noted in the following that, for the majority of the former, Takenaka's reports agree with those of others. Kehr and Smith (1952) have reported nine new hybrids,

primarily involving *N. bigelovii, N. debneyi, N. clevelandii, N. glutinosa, N. glauca* and *N. plumbaginifolia;* they described these hybrids morphologically but did not report their cytological behavior. However, from six of these as well as from ten hybrids previously known, amphiploids were produced by colchicine treatment, and the extent of pairing and meiotic irregularities therein noted. In addition, Kehr and Smith (*l.c.*) made eighteen triple genom combinations by crossing amphiploids with a third, unrelated, species, and five quadruple genom combinations by crossing unrelated amphiploids. In two cases they were successful in obtaining plants combining diploid complements of three species: *N. bigelovii, N. debneyi* and *N. tabacum; N. debneyi, N. glauca* and *N. sanderae.* The former multiple allopolyploid ($2n=144$) is of interest because it combines diploid genoms of three 24-paired species of widely different geographic origin: *N. bigelovii* from North America, *N. debneyi* from Australia and *N. tabacum* from South America.

Kehr and Smith reported that the quadruple hybrid made by crossing this allopolyploid plant, containing diploid genoms of *N. bigelovii, N. debneyi* and *N. tabacum,* with pollen of *N. glutinosa* possessed 84 chromosomes as the $2n$ number and showed 3 to 6 bivalents at first meiotic metaphase, whereas the total number of bivalents occurring in four of the six F_1 diploid combinations of these four species was 2 to 14. This comparison was interpreted as indicating that there are a number of chromosomes with homologous segments in common among three or four of the species and that, because of random synapsis of these segments in the quadruple hybrid, fewer bivalents were produced than the total in the F_1's. This provides significant evidence of residual homologies in species of four sections of two subgenera, in the evolutionary development of which there has been wide morphological and geographic divergence.

Further, these studies of multiple hybridizations demonstrate an altered compatibility shown by an amphiploid, as compared with its component species, in crosses with other species. For example, whereas neither *N. debneyi* nor *N. tabacum* can be crossed readily with *N. langsdorffii,* the cross (amphiploid *N. debneyi* x *N. tabacum*) x *N. langsdorffii* was easily made and produced viable seed (Kehr and Smith, *l.c.*). The evolutionary significance of such experimental evi-

dence that amphiploidy may extend the range of hybridity beyond that possible on the diploid level is obvious.

Kehr and Smith found no evidence of a limit in tolerance to increase in chromosome number and concluded that it is primarily a lack of specific genom divergence within the genus, not chromosome number, which imposes the limitation to constructing complex fertile allopolyploids. Further work by Smith (1958) on the multiple allopolyploid *N. bigelovii-debneyi-tabacum* ($n=72$) supports this conclusion. The allopolyploid was originally produced by crossing the amphiploid *N. debneyi-tabacum* ($n=48$) with *N. bigelovii* ($n=24$) and doubling the chromosome number with colchicine. Cultures were continued through the S_{10} generation by self pollination and selection for three distinct races. Each race became established at a different high aneuploid number ($n=50$ to 60) and, despite persisting meiotic irregularities, showed high fertility and distinctive morphology.

In what follows, cytological data bearing upon the taxonomic organization of the genus *Nicotiana,* those detailed in the monograph and those more recently reported, will be summarized and interpreted, section by section, in terms of relationships, origins and mechanisms of evolution, and with reference to a chart (Fig. 1,) which shows the putative phylesis of the major taxonomic subdivisions of the genus.

In section PANICULATAE each of the seven species is 12-paired and, except in *N. benavidesii* and *N. glauca,* all centromeres are median or submedian. *N. benavidesii* differs only in possessing two chromosomes with subterminal centromeres, while *N. glauca* is extreme in having ten such chromosomes. The latter species is correspondingly set apart from the remainder of the section in the meiotic behavior of its F_1 hybrids. Of thirteen F_1 intra-PANICULATAE hybrids, nine, none of which involves *N. glauca,* show complete or almost complete pairing, and four, in all of which *N. glauca* is a parent, show minimum pairing. That such pairing as does occur in the latter hybrids is allosyndetic is indicated by the heteromorphic character of the bivalents, for example, in F_1 *N. paniculata* x *N. glauca,* combining two species in which the chromosomes are extreme in size distinctions. Pairing in this hybrid ranges from zero in 45% to 3 pairs in only 3% of the pollen mother cells. Similarly, in the F_1 hybrids combining *N. glauca* with *N. raimondii, N. benavidesii* and *N. solanifolia,* respectively, pairing ranges from zero (as the mode) to 4 pairs (rarely). Thus,

it appears that only a few chromosomes in the genom of *N. glauca* possess segments which are homologous with segments in the genoms of the remaining species of the section. This suggests that chromosome reorganization has contributed to the isolation of *N. glauca* cytologically from the species with which it is most closely associated morphologically.

Pairing relations in twenty-five F_1 intersectional hybrids, in each of which a member of section PANICULATAE is a partner, contribute as follows to the validity of the taxonomic organization of the genus. Three F_1 hybrids between members of section PANICULATAE and *N. rustica* show "Drosera scheme" pairing, thereby demonstrating the relation of a member of the former section to the amphiploid origin of the latter species. In F_1 *N. rustica* x *N. paniculata*, Takenaka (1956c) has reported a much wider range in pairs (2 to 12) and in trivalents (0 to 10) than the 12 bivalents and occasional trivalent observed by Goodspeed (1954) and by Kostoff (1943). Ten hybrids earlier reported, and two more recent ones (cf. p.), combining members of section PANICULATAE with members of subgenus TABACUM show low or high variable pairing range, thus indicating more genic homology than is expressed in a corresponding number of hybrids between members of section PANICULATAE and members of subgenus PETUNIOIDES, where pairing is of the minimum category. This is consistent with the source of subgenus TABACUM in the same ancestral complex (cestroid) as that from which subgenus RUSTICA stems (cf. Fig. 1), as contrasted with the basic origin of subgenus PETUNIOIDES in a distinct ancestral complex (petunioid). Noteworthy exceptions to the minimum pairing usually found in F_1 hybrids between members of section PANICULATAE and members of sections of subgenus PETUNIOIDES are those with members of section UNDULATAE, where somewhat higher pairing reflects the bridging nature of the latter section between products of the two ancestral complexes (cf. Fig. 1; also Goodspeed, 1954, pp. 310, 312). Similar meiotic behavior reported by others for F_1 hybrids between members of sections PANICULATAE and ALATAE may be interpreted as a reflection of the introduction by hybridization of prepaniculatoid elements into the basically petunioid progenitor of section ALATAE (cf. Fig. 1). However, such extensive pairing as Kostoff (*l.c.*) found in F_1 *N. glauca* x *N. alata* (6-9 pairs), in F_1 *N. glauca* x *N. langsdorffii* (2-8, 3-9 in the reciprocal) and in F_1

N. glauca x *N. longiflora* (6-10) is difficult to explain, particularly in view of Takenaka's (1953b) observation of 0-7 pairs in the first named hybrid, 1-6 in *N. longiflora* x *N. glauca* and 0-4 in *N. glauca* x *N. plumbaginifolia.* The same low range of pairing was also reported for the last named hybrid by Ramanujam and Joshi (1942) and by Goodspeed (*l.c.*).

Thus, the taxonomic identity of section PANICULATAE, its intrasectional relationships and its relationships with other taxonomic entities, all based largely upon morphological and distributional considerations, are fully supported and documented by cytological data. Similarly supported are the taxonomic identities of the remaining divisions of the genus.

Of seventeen F_1 interspecific hybrids involving *N. rustica,* in addition to the three above mentioned which unite *N. rustica* and members of section PANICULATAE, only F_1 *N. rustica* x *N. undulata* shows "Drosera scheme" pairing, thus demonstrating the affinity of *N. undulata* with the other progenitor of *N. rustica.* In F_1 *N. rustica* x *N. wigandioides* pairing is low variable, evidence of one common and one distinct 6-paired progenitor in the origin of *N. undulata* and *N. wigandioides,* both of section UNDULATAE. A similar extent of pairing in F_1 *N. rustica* x *N. tabacum* (0-7, mode 3) is interpreted as evidence of homologies between the paniculatoid subgenom of *N. rustica* and the tomentosoid subgenom of *N. tabacum,* homologies based on the origin of both elements in the cestroid complex (cf. Fig. 1). Takenaka (1955b) reported from 1 to 10 pairs in this hybrid, while Kostoff (*l.c.*) found 5-24 pairs.

Meiotic behavior in eight F_1 intra-TOMENTOSAE hybrids reveals close relationships among *N. otophora, N. tomentosa, N. tomentosiformis* and *N. setchellii* and a lesser affinity between each of these species and *N. glutinosa,* the fifth member of the section, corresponding to the morphological and karyotypic divergence of the latter from the remainder of the section.

Some thirty F_1 intersectional, including intersubgeneric, hybrids involving members of section TOMENTOSAE express, through their meiotic behavior, various degrees of relationship between the species and groups of species concerned. Thus, appreciable pairing in F_1 hybrids between members of sections PANICULATAE and TOMENTOSAE is taken to reflect the origin of the two sections as separate lines of

divergence from the same ancestral complex (cestroid). Somewhat higher pairing in F_1 *N. benavidesii* x *N. glutinosa* than in other hybrids between members of sections PANICULATAE and TOMENTOSAE may support the postulate, based upon certain morphological resemblances in the two species, that their evolution involved introgressive hybridization on the 12-paired level between products of the two divergent lines.

Of the five F_1 hybrids combining members of section TOMENTOSAE with *N. tabacum,* four show the "Drosera scheme" pairing characteristic of an F_1 between an amphiploid and a diploid species related to one of its progenitors; pairing in F_1 *N. tabacum* x *N. glutinosa,* on the other hand, ranges from zero to 11 with mode at 5 pairs, again reflecting the distinction of *N. glutinosa* from other members of its section.

Eight F_1 hybrids combining a member of section TOMENTOSAE and one of either section UNDULATAE or section TRIGONOPHYLLAE show high variable pairing except when the tomentosoid species is *N. glutinosa,* and even in these hybrids pairing is appreciable. This extent of pairing is interpretable as reflecting the bridging character of sections UNDULATAE and TRIGONOPHYLLAE, combining elements stemming from the two ancestral complexes. In the remaining F_1 intersubgeneric hybrids involving members of section TOMENTOSAE, pairing is either minimum or low variable, the latter notably in those combining a tomentosoid species with *N. sylvestris* (cf. also Takenaka, 1955c). This considerable amount of pairing in F_1 hybrids, uniting the two 12-paired species related to the amphiploid origin of *N. tabacum,* is significant in the interpretation of relationships based on pairing in F_1 hybrids combining *N. tabacum* with other 12-paired species. In these latter hybrids autosyndesis in the *N. tabacum* genom cannot be overlooked as possibly accounting for at least a few pairs. Such interpretation is consistent with the observation of from one to several bivalents in certain *N. tabacum* haploids (Goodspeed, 1954; Takenaka and Tanaka, 1956) and frequent trivalents in all F_1 hybrids combining *N. tabacum* with a member of section TOMENTOSAE or with *N. sylvestris.*

Among the twenty-two F_1 hybrids with *N. tabacum* as a parent, apart from the five uniting *N. tabacum* with a member of section TOMENTOSAE or with *N. sylvestris* where "Drosera scheme" pairing

prevails, the majority show from low to high variable pairing. While autosyndesis may be the source of a certain amount of pairing in each case, the fact that in four F_1 hybrids only minimum pairing has been observed (Kostoff, 1943) suggests that the amount of autosyndesis is limited and that at least a portion of the pairing expresses residual homologies between one or the other subgenom of *N. tabacum* and the genom of the other species involved in the hybrid.

By contrast with an earlier report (Kostoff, *l.c.*) of 5 to 9 pairs in F_1 *N. tabacum* x *N. alata,* Takenaka (1956a) reported 1 to 6 pairs (mode 3-4) in one hundred and twenty-five pollen mother cells analyzed, and attributed a portion of the pairing observed to autosyndesis resulting from intersubgenomal homologies in *N. tabacum.* It is to be noted that a portion of the pairing could be interpreted as expressing residual relationship between *N. alata* and the *N. sylvestris*-related subgenom of *N. tabacum,* as has been that in F_1 *N. tabacum* x *N. plumbaginifolia,* although certainly the evolutionary processes which have operated in speciation in section ALATAE—including reduction in chromosome number from 12 to 10 and 9, alteration in chromosome morphology to increase the proportion of chromosomes with subterminal centromeres, and introduction of additional petunioid characters presumably by hybridization—have greatly reduced homologies between present 9- and 10-paired species and the related subgenom in *N. tabacum.* In F_1 *N. tabacum* x *N. glauca,* Takenaka (1953c) reported somewhat higher pairing (3-10 pairs with some trivalents) but lower than Kostoff's report of 9-12 pairs. It is possible that genetic differences in the races of *N. tabacum* used by the various investigators are in part responsible for these varying reports of degree of pairing. Takenaka's observations (*l.c.,* 1956c) of 1-7 pairs in F_1 *N. glutinosa* x *N. tabacum,* 0-5 in *N. tabacum* x *N. solanifolia* and 0-6 in *N. debneyi* x *N. tabacum* are in general agreement with those of Goodspeed and Kostoff.

Of the five F_1 hybrids reported by Takenaka and his associates for the first time, three involve *N. tabacum* as one parent. Hu (1956) found in F_1 *N. tabacum* x *N. langsdorffii* 5 to 12 pairs (mode 11 pairs), pairing which was interpreted as showing "almost complete homology between the genom of *N. langsdorffii* and one subgenom of *N. tabacum*". This appears inconsistent with Takenaka's report (1955a) for *N. tabacum* x *N. alata,* particularly in view of the com-

plete pairing (9 pairs) in F_1 *N. alata* x *langsdorffii*, commented upon in the same article.

Takenaka's report (1955b) of zero to 11 pairs (mode 5-6) in F_1 *N. tabacum* x *N. trigonophylla* is consistent with Goodspeed's report (1954) of zero to 10 pairs (mode 4) in F_1 *N. tabacum* x *N. palmeri* and with the extent of pairing in F_1 hybrids between either *N. palmeri* or *N. trigonophylla* and members of section TOMENTOSAE (Goodspeed, *l.c.*). This pairing presumably reflects residual homologies between the tomentosoid subgenom in *N. tabacum* and the genom of *N. trigonophylla*, evidence of relationship on the 6-paired level between progenitors of sections TRIGONOPHYLLAE and TOMENTOSAE (cf. Fig. 1).

In F_1 *N. undulata* x *N. tabacum*, Takenaka (*l.c.*) observed zero to 8 pairs (mode 3-5) and attributed pairing largely to autosyndesis between the two subgenoms of *N. tabacum*. However, it is to be noted that a portion of the observed pairing may reflect some residual homology from the 6-paired level between the genom of *N. undulata* and the tomentosoid subgenom of *N. tabacum*.

Takenaka (1955c) also reported for the first time, the amount of pairing in two F_1 hybrids between members of sections PANICULATAE and TOMENTOSAE: in F_1 *N. paniculata* x *N. otophora*, 3 to 10 pairs (mode 4-5); in F_1 *N. glauca* x *N. otophora*, 1 to 9 pairs (mode 4-5), an extent of pairing interpretable as a reflection of relationship on the 6-paired level between the progenitors of the two sections.

Section UNDULATAE is unique in the genus in containing a 24-paired amphiploid (*N. arentsii*) and the two 12-paired species which represent its progenitors (*N. undulata* and *N. wigandioides*). Although both of the 12-paired species are fundamentally allied with members of subgenus PETUNIOIDES, in external morphology and in karyotype each shows some affinity with a different subgenus, *N. undulata* with subgenus RUSTICA and *N. wigandioides* with subgenus TABACUM. Evidence from meiotic behavior in F_1 interspecific hybrids confirms this affinity and refers its origin to 6-paired ancestry. Thus, it is postulated that the two species had one common 6-paired parent in the pre-PETUNIOIDES aggregate which combined with two distinct 6-paired races derived from the cestroid complex—with a 6-paired race in the pre-RUSTICA aggregate ultimately to produce *N. undulata*, and again with a 6-paired race in the pre-TABACUM aggregate to produce *N. wigandioides*. The confirm-

ing meiotic behavior in F_1 intra- and intersectional hybrids is as follows: F_1 *N. undulata* x *N. wigandioides* shows pairing range of 2 to 9 with mode at 5 pairs, and in the F_1 hybrids combining each 12-paired species with their amphiploid *(N. arentsii)* frequent multivalents are included in the essentially "Drosera scheme" pairing which characterizes them. Eleven F_1 intersectional and intersubgeneric hybrids demonstrate the bridging nature of the 12-paired species of section UNDULATAE, *N. undulata* to subgenus RUSTICA, *N. wigandioides* to subgenus TABACUM. Low variable pairing range in F_1 hybrids combining *N. undulata* with most of the members of section PANICULATAE, and extensive multivalent formation in F_1 *N. rustica* x *N. undulata,* reflect residual homologies of *N. undulata* with members of section PANICULATAE, while range of 1 to 9 pairs in the one F_1 hybrid combining *N. wigandioides* with a member of section TOMENTOSAE indicates residual homologies in the two genoms consistent with the postulated origin of *N. wigandioides.*

The close affinity of the two species of section TRIGONOPHYLLAE is expressed in complete pairing in the F_1 combining them, while a certain genetic distinction between them is suggested by some differences in meiotic behavior in F_1 hybrids combining each of the two species with the same species *(N. setchellii)* and thus supports their taxonomic separation. Six F_1 hybrids between members of sections TOMENTOSAE and TRIGONOPHYLLAE indicate the presence of sufficient homology in the genoms of species of the two sections to show a maximum of 7 to 11 pairs and a mode of approximately 6 pairs. Such extent of pairing is interpreted as evidence of relationship through a common progenitor on the 6-paired level, a postulated relationship which is supported by pairing range of zero to 10 pairs (mode 4) in F_1 *N. tabacum* x *N. palmeri.*

Of eleven F_1 intra-ALATAE hybrids, four combining 9-paired species and one between the two 10-paired species, show complete association; of the six combining 9- and 10-paired species, all but two show association of 9 pairs. Considerable multivalent formation in the latter hybrids and even more extensive multivalent formation in F_1 hybrids between the 9-paired species, suggest that chromosome reorganization has been a significant factor in the evolution of members of section ALATAE (cf. also Takenaka, 1955d). By contrast, the remaining two hybrids which combine each of the 10-paired species with *N. bon-*

ariensis (9-paired) show minimum pairing. In view of the complete chromosome association in F_1 hybrids combining either parent with *N. alata* or with *N. langsdorffii,* this almost complete lack of pairing is attributed to the operation of some physiological or genetic factor rather than to lack of homology.

F_1 intersectional hybrids involving a member of section ALATAE in general confirm relationships already commented upon: "Drosera scheme" pairing in F_1 *N. tabacum* x *N. sylvestris* indicates the relationship of the latter species to one progenitor of the former; somewhat above minimum pairing in F_1 hybrids combining *N. sylvestris* with members of section TOMENTOSAE indicates a degree of residual homology in their genoms based upon hybridization on the 12-paired level between their progenitors. Absence of more than minimum pairing in F_1 intrasubgeneric hybrids involving a 9- or 10-paired member of section ALATAE may be a product of the extensive chromosome reorganization which has been a factor in the evolution of these species. As in section REPANDAE, there has been no direct cytological confirmation of the morphological evidence of participation of an early 12-paired alatoid race in the amphiploid origin of section BIGELOVIANAE.

Two of the three 24-paired species composing section REPANDAE, *N. stocktonii* and *N. repanda,* have been combined, respectively, with the third species, *N. nesophila,* in F_1 hybrids, both of which show approximately complete pairing with some multivalent formation; thus is indicated the close relationship of the three species, with chromosome reorganization a probable factor in their divergence, presumably from a common 24-paired entity. That one 12-paired progenitor of such 24-paired amphiploid is related to membes of section TRIGONOPHYLLAE, at least on the 6-paired level, is apparent in the presence of up to 10 pairs in F_1 *N. repanda* x *N. palmeri.* A limited part of the pairing and the occurrence of from 1 to 3 trivalents in over one-half of the pollen mother cells in this hybrid may be attributed to chromosome reorganization. However, certain of the intragenomal homologies so expressed may be residual between the subgenoms of *N. repanda,* since, although cytological evidence of the affinities of the other 12-paired progenitor of section REPANDAE is lacking, morphological evidence relates it to section ALATAE.

The one intra-NOCTIFLORAE hybrid studied, F_1 *N. noctiflora* x *N.*

petunioides, shows complete pairing, while two F_1 hybrids combining *N. noctiflora* with species of section PANICULATAE show minimum pairing. By contrast, an F_1 hybrid between *N. petunioides* and a member of section ACUMINATAE, *N. corymbosa,* shows a pairing range of 3 to 10 with mode at 7 pairs. Such pairing is interpreted as due, in part, to considerable preservation of initial homology from 6-paired ancestry common to both sections in the petunioid complex which, in view of present distributional overlapping of the two sections, may well have been increased through hybridization after attainment of the 12-paired level.

In four F_1 intra-ACUMINATAE hybrids pairing is high to complete: in two combining *N. acuminata* with *N. corymbosa* and *N. pauciflora,* respectively, 12 pairs without multivalent formation indicate a high degree of structural and genic equivalence of the two genoms, corresponding to a close taxonomic relationship between the two species. The other two hybrids, F_1 *N. attenuata* x *N. miersii* and F_1 *N. corymbosa* x *N. linearis,* show a wider range of pairing with a mode approaching 12 pairs and some meiotic evidence of the chromosome distinctions which are also shown karyotypically.

Sixteen F_1 intersectional hybrids involve members of section ACUMINATAE. Apart from one, F_1 *N. corymbosa* x *N. petunioides,* already referred to as showing high variable pairing, only those four combining species of the section with members of section BIGELOVIANAE (24-paired) show more than minimum pairing. In the four uniting *N. attenuata* and *N. miersii* each with *N. bigelovii* and *N. clevelandii,* respectively, "Drosera scheme" pairing, with extensive multivalents and chromosome association, in some pollen mother cells, in excess of the equivalent of 12 pairs, indicates, at once: the close relationship of the two 24-paired species of section BIGELOVIANAE, *N. bigelovii* and *N. clevelandii;* the relationship of members of section ACUMINATAE (12-paired) to one progenitor of section BIGELOVIANAE; the presence of additional intragenomal homologies, presumably in large part residual in the two subgenoms of the 24-paired species which apparently arose by amphiploidy uniting progenitors of sections ACUMINATAE and ALATAE.

Fifteen additional hybrids combine members of section BIGELOVIANAE with members of other sections; all show an extent of pairing consistent with relationships of the species concerned. The occurrence

of zero to 10 pairs (mode 4) in F_1 *N. bigelovii* x *N. tabacum* is interpreted, in part, as autosyndesis in one or both genoms, and in part, as allosyndesis between members of the *N. sylvestris*-related subgenom of *N. tabacum* and the alatoid subgenom of *N. bigelovii*. Two F_1 hybrids combining *N. nudicaulis* with *N. bigelovii* and *N. clevelandii*, respectively, show high variable pairing, with mode at 8 and 6, and extensive multivalent formation. Although a limited amount of pairing may be autosyndetic in the *N. bigelovii* genom, the majority of it is taken to indicate intergenomal homologies presumably inherited by the three species from a 12-paired acuminatoid progenitor.

All other F_1 intersectional hybrids involving *N. nudicaulis* show minimum pairing except F_1 *N. nudicaulis* x *N. trigonophylla* or x *N. palmeri*, which show "Drosera scheme" pairing, indicating that the other progenitor in the amphiploid origin of *N. nudicaulis* was related to section TRIGONOPHYLLAE.

Twenty-nine F_1 intra-SUAVEOLENTES hybrids combine twelve of the fourteen species which are known cytologically, and contribute significantly to the interpretation of relationships in a section which is outstanding in the genus in complete geographic isolation, in possession of an aneuploid series of many chromosome numbers, in character of meiotic behavior and in external morphology. All of the twenty-nine hybrids show high variable to "Drosera scheme" or complete pairing (Kostoff, 1943; Takenaka, 1953d; Goodspeed, 1954) and thus give evidence of relationship based on high degree of homology. Evidence that chromosome reorganization has been a significant factor in the evolution of members of section SUAVEOLENTES is provided by the extensive multivalent formation which occurrs in all but four of the twenty-nine hybrids in a large percentage of pollen mother cells, in two hybrids in every pollen mother cell. A portion of this multivalent formation may reflect residual homologies between two 12-paired progenitors of section SUAVEOLENTES, since this aneuploid series is interpreted as having originated through reduction following the production of a series of amphiploids involving 12-paired alatoid, acuminatoid and noctifloroid progenitors (cf. Fig. 1). However, twenty-nine intersectional hybrids combining members of section SUAVEOLENTES with members of other sections, particularly ALATAE and ACUMINATAE, show only minimum pairing (Kostoff, *l.c.;* Takenaka, *l.c.;* Goodspeed, *l.c.*). Apparently few homologies between the modern sections

most closely related to the ancestry of section SUAVEOLENTES and current members of that section have survived its long established geographic isolation, the hybridization and the extensive chromosome reorganization which have been a part of its evolution.

CONCLUSIONS

The cytogenetic contributions to the taxonomic picture of the genus *Nicotiana* consist of evidence from chromosome number, morphology and meiotic behavior. To a rather remarkable degree the conclusions drawn from this cytogenetic evidence confirm those from morphology and distribution and go significantly further in providing a basis for postulates concerning species origins and evolution.

As already briefly noted, of the evidence basic for those postulates, that from meiotic behavior has proved to be particularly important. Of two hundred and twenty F_1 interspecific hybrids cytologically analyzed, approximately 90% of all intrasectional combinations show complete or nearly complete pairing. By contrast, approximately 90% of intersectional hybrids and a much greater percentage of intersubgeneric hybrids show only minimum pairing (except those between 24-paired amphiploids and 12-paired species related to their origin). It follows that in 10% of intrasectional and in an equivalent percentage of intersectional hybrids, extent of pairing does not correspond to degree of taxonomic relationship. Failure of complete pairing in certain intrasectional hybrids is assumed to be a product of chromosome reorganization which has disrupted pairing blocks without creating major alterations in external morphology. The occurrence of more extensive pairing than expected in 10% of F_1 intersectional hybrids may be referable to relationship on an earlier evolutionary level.

Fig. 1 shows the putative phylesis of the major taxonomic subdivisions of the genus, including its fourteen sections. The sixty species composing those sections are charted in respect to derivations and relationships in the monograph (Goodspeed 1954, pp. 310, 312).

Three major arcs, set apart by solid lines, represent three evolutionary levels: arc 1, the pregeneric level; arc 2, the ancestral *Nicotiana* level; arc 3, the modern *Nicotiana* level. Arcs 1 and 2 provide the hypothetical background for the current expression of the genus in arc 3; hypothetical derivations are shown by dotted lines, demonstrable ones by solid lines.

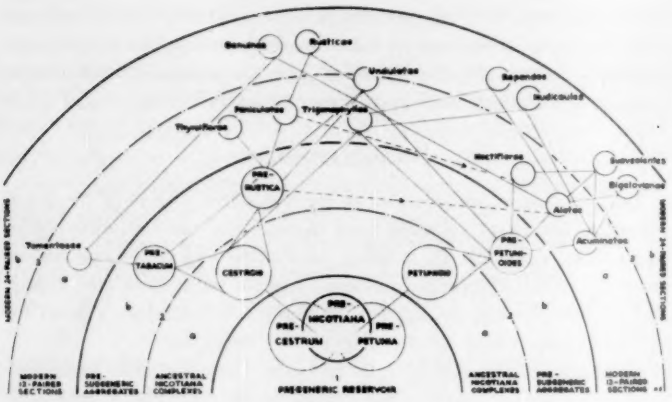

Fig. 1.

Origins and Relationships of the Fourteen Sections of the Genus *Nicotiana*.

Arc 1—For our purpose we need recognize, among the differentiating Solanaceous elements, only those which were the progenitors of the modern genera *Cestrum* and *Petunia,* which today show a certain relationship to *Nicotiana.* Each pregeneric element, pre-*Cestrum,* pre-*Petunia* and pre-*Nicotiana,* is given identity in the reservoir by an enclosing circle. Based upon morphological expression in the three present-day genera, the postulated relative evolutionary status of the three pregeneric elements, from the more primitive (pre-*Cestrum*) to the more advanced (pre-*Petunia*) with pre-*Nicotiana* intermediate, is symbolized by the relative position, left to right, of the three circles. Overlapping of the circles further emphasizes the intermediate relationship of pre-*Nicotiana* to the other two: certain elements in pre-*Nicotiana* express some pre-*Cestrum*-like characters, while others show pre-*Petunia*-like characters.

Arc 2—The ancestral *Nicotiana* level is divided into two sublevels: a) contains the two ancestral complexes of 6-paired races, the cestroid and the petunioid, directly derived from the pre-*Nicotiana* circle, the former from the area bordering on pre-*Cestrum,* the latter from that bordering on pre-*Petunia;* b) contains three "presubgeneric" aggregates, hypothetical precursors of modern subgenera, predominantly 12-paired, derived via amphiploidy as shown by two converging lines—pre-RUSTICA and pre-TABACUM from distinct 6-paired elements in the

cestroid complex, pre-PETUNIOIDES from the petunioid complex. Not shown is the direct derivation of certain postulated 6-paired components of the three presubgeneric aggregates.

Arc 3—The modern, like the ancestral, *Nicotiana* level is divided into two sublevels: a) contains the 12-paired constituents of the genus; b) contains the 24-paired amphiploid constituents. Each circle represents a section in the present taxonomic organization of the genus, certain of them on the 12-paired level, others on the 24-paired and one in part on each level. One section (ALATAE) on the 12-paired level contains as well as a 12-paired species, an aneuploid series (9- and 10-paired) below that number; another (SUAVEOLENTES) contains 24-paired species and an aneuploid series derived therefrom. For sections PANICULATAE, THYRSIFLORAE, TOMENTOSAE and ACUMINATAE, direct derivation is shown by a single line from a presubgeneric aggregate; for others (ALATAE and NOCTIFLORAE) hybrid origin is shown by a dash line and a solid line, the latter indicating introgression; for still others (UNDULATAE and TRIGONOPHYLLAE) postulated amphiploid origin is shown by the meeting of two solid lines representing 6-paired elements of different presubgeneric aggregates. The amphiploid origin of each 24-paired section is shown by the meeting of two solid lines of derivation from the 12-paired level.

The composite picture expressed in the chart involves amphiploidy superimposed upon amphiploidy to produce a 6-12-24-paired sequence. On each successive level genetic differentiation progressed by means of introgressive hybridization and alterations in chromosome organization and genic constitution, accompanied by segregation, selection and isolation.

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Printed in U. S. A.
Publication Office:
Interstate Printing Corporation
400 Watchung Avenue
Plainfield, N. J.